HEI

RESEARCH REPORT

HEALTH
EFFECTS
INSTITUTE

Number 113
February 2003

Benzene Metabolism in Rodents at Doses Relevant to Human Exposure from Urban Air

Kenneth W Turteltaub and Chitra Mani

Includes a Commentary by the Institute's Health Review Committee

HEALTH
EFFECTS
INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate matter) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 200 projects at institutions in North America and Europe and has published over 130 research reports.

Typically, HEI receives half its funds from the US Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the United States. Occasionally, funds from other public and private organizations either support special projects or provide resources for a portion of an HEI study. Regardless of funding sources, HEI exercises complete autonomy in setting its research priorities and in reaching its conclusions. An independent Board of Directors governs HEI. The Institute's Health Research and Health Review Committees serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded studies are made available as Research Reports, which contain both the Investigators' Report and the Review Committee's evaluation of the work's scientific quality and regulatory relevance.

HEI STATEMENT

Synopsis of Research Report 113

Benzene Metabolism in Rodents at Doses Relevant to Human Exposure

Human exposure to high levels of benzene is associated with development of leukemia and other blood disorders, but the effects of exposure to low levels of benzene are not well understood. In the 1990s, HEI initiated its Air Toxics Research Program to address uncertainties about health effects of ambient levels of benzene and other air toxics derived from mobile and other sources. One of the program's goals was to develop methods sensitive enough to measure benzene metabolism at low exposure levels. Such sensitivity is important because one or more benzene metabolites are thought to be responsible for benzene's toxic effects. In addition, understanding benzene metabolism at low exposure levels is critical to benzene risk assessment because the shape of the dose-response curve at low concentrations is not yet resolved.

APPROACH

HEI funded Dr Kenneth Turteltaub to investigate benzene metabolism in rodents over a hundred million–fold dose range. This range encompassed concentrations close to those of human ambient exposure, generally 1 to 10 parts per billion. Turteltaub and his colleague, Chitra Mani, administered radioactive benzene to mice and rats and subsequently analyzed bone marrow, liver, urine, and plasma from these animals. In most experiments, the investigators injected animals intraperitoneally with radioactive benzene, but in some experiments they exposed animals to ^{14}C-labeled benzene via inhalation. After exposure, the investigators coupled high-performance liquid chromatography (HPLC; to separate benzene metabolites) with the novel and sensitive technique accelerator mass spectrometry (to measure ^{14}C) in order to measure low levels of metabolites. Accelerator mass spectrometry was developed by nuclear physicists to measure low levels (10^{-15} to 10^{-18} molar) of long-lived isotopes such as ^{14}C.

RESULTS AND INTERPRETATION

In this innovative study of benzene metabolism, Turteltaub and Mani detected dose-dependent formation of benzene metabolites in plasma, bone marrow, and liver of mice over a wide range of doses (5 ng/kg to 500 mg/kg). Benzene metabolites, including DNA and protein adducts, were detected at levels 100 times lower than had been found in previous studies.

Even at low benzene exposure concentrations, the investigators detected higher levels of benzene metabolites in mouse and rat bone marrow and liver than in plasma. This finding indicates that benzene reaches tissues and is metabolized there, even at levels close to those to which humans are exposed in ambient air. In addition, Turteltaub and Mani found that the levels of DNA and protein adducts detected in bone marrow and liver in different rodents generally correlated well with the ability of benzene to induce tumors in that species or strain. This result suggests that the formation of adducts may be an early marker of benzene carcinogenicity.

All doses of benzene produced a similar pattern of metabolites in mouse urine, suggesting that the pattern of benzene metabolism is similar at widely disparate concentrations. This finding is of interest because other studies have suggested that the pattern of benzene metabolites differs depending on the benzene concentration to which animals are exposed. Such differences in metabolism of benzene could affect the shape of the exposure-response curve. However, Turteltaub and Mani's results are difficult to compare with previous studies: Although the current study has greater intrinsic analytic sensitivity than previous studies, it did not detect a metabolite previously found in the urine of rodents exposed to benzene.

Although results of the current study show the potential of accelerator mass spectrometry coupled with HPLC, they also illustrate the drawbacks. First,

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Drs Kenneth W Turteltaub and Chitra Mani at Lawrence Livermore National Laboratory, Livermore CA, and the University of California, San Francisco CA. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

in the current study, urine from mice exposed to radioactive benzene contained a large peak of radioactivity that could not be identified by HPLC. The investigators did not look for this material in plasma or bone marrow; thus, the peak might also have been present in samples from these tissues, with an uncertain impact on the results. This unidentified radioactive material may be a contaminant of the radioactive material used in the assays, a previously unidentified metabolite, or the decomposition product of a known benzene metabolite.

Second, the technique requires administering radiolabeled benzene to the study animals. Although the method uses extremely low levels of radioactive benzene, such an approach is not broadly applicable for controlled exposure studies with humans because benzene is classified as a known human carcinogen. Third, this study indicates the potential influence on results of varying methods of biomarker collection, storage, and processing. In the current study, glucuronidase inhibitors were not added to urine samples, which possibly resulted in the degradation of a major metabolite, hydroquinone glucuronide, that was detected in other studies of benzene metabolism.

Even given these challenges, Turteltaub and Mani provided important information about benzene metabolism at the lowest end (5–500 ng/kg body weight) of the range of benzene doses tested: the dose-response curve for metabolite formation was flatter than that of higher benzene doses but was above zero. This result indicates that metabolism of benzene to activated metabolites occurs even at very low doses. It further suggests, but does not show conclusively, that the dose-response curve for benzene in mice lacks an obvious threshold at the lowest exposure levels evaluated. This finding may have important ramifications for understanding the human response to low-level benzene exposures. Further studies are required to resolve the shape of the dose-response curve for humans at these low benzene levels.

 Cameographics, Union ME, Compositor. Printed at Capital City Press, Montpelier VT.
Library of Congress Catalog Number for the HEI Report Series: WA 754 R432.
The paper in this publication meets the minimum standard requirements of the ANSI Standard Z39.48-1984 (Permanence of Paper) effective with Report 21 in December 1988; and effective with Report 92 in 1999 the paper is recycled from at least 30% postconsumer waste with Reports 25, 26, 32, 51, 65 Parts IV, VIII, and IX, 91 and 105 excepted. These excepted Reports are printed on acid-free coated paper.

HEALTH
EFFECTS
INSTITUTE

CONTENTS
Research Report 113

Benzene Metabolism in Rodents at Doses Relevant to Human Exposure from Urban Air

Kenneth W Turteltaub and Chitra Mani

Molecular and Structural Biology Program/Center for Accelerator Mass Spectrometry, Lawrence Livermore National Laboratory, Livermore, California, and Department of Pharmaceutical Chemistry, University of California, San Francisco, California

HEI STATEMENT

This Statement is a nontechnical summary of the Investigators' Report and the Health Review Committee's Commentary.

INVESTIGATORS' REPORT

When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three ouside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in selecting or managing the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, if necessary, revise the report.

Continued

Research Report 113

COMMENTARY Health Review Committee

The Commentary about the Investigators' Report is prepared by the HEI Health Review Committee and Staff. Its purpose is to place the study into a broader scientific context, to point out its strengths and limitations, and to discuss remaining uncertainties and implications of the findings for public health.

RELATED HEI PUBLICATIONS

Publishing History: This document was posted as a preprint on *www.healtheffects.org* and then finalized for print.

Citation for whole document:

Turteltaub KW, Mani C. 2003. Benzene Metabolism in Rodents at Doses Relevant to Human Exposure from Urban Air. Research Report 113. Health Effects Institute, Boston MA.

When specifying a section of this report, cite it as a chapter of the whole document.

INVESTIGATORS' REPORT

Benzene Metabolism in Rodents at Doses Relevant to Human Exposure from Urban Air

Kenneth W Turteltaub and Chitra Mani

ABSTRACT

Benzene is both an environmental pollutant and a component of cigarette smoke, gasoline, and automotive emissions. Although occupational exposure to benzene has been shown to cause blood disorders and cancer in humans, the potential health effects resulting from exposure to low levels of benzene are not known. The goals of this project were to determine how well benzene is metabolized and to assess its binding to macromolecules in rodents at doses more closely mimicking human environmental exposure.

To determine whether genotoxic metabolites of benzene are produced at environmental exposure levels, various doses of ^{14}C-benzene were given intraperitoneally to male B6C3F$_1$ mice at doses from 5 ng/kg to 500 mg/kg body weight. Samples of urine, plasma, liver, and bone marrow were taken at selected times up to 48 hours after exposure. Individual benzene metabolites in the samples were measured by accelerator mass spectrometry (AMS*). Metabolites were quantified by determining the area under the curve (AUC) for 24 to 48 hours. The major metabolites found in urine were an unidentified radiolabeled metabolite, phenyl sulfate, phenyl glucuronide, and muconic acid (an indicator of muconaldehyde formation). The major metabolites found in plasma, liver, and bone marrow samples were muconic acid and hydroquinone. Only liver showed a dose response for hydroquinone and muconic acid.

The kinetics of both DNA and protein adduct formation were assessed over 48 hours at a ^{14}C-benzene dose of 5 µg/kg body weight. A dose-response study was then conducted using ^{14}C-benzene doses from 5 ng/kg to 500 mg/kg body weight in B6C3F$_1$ mice. Adduct levels were determined by AMS in liver and bone marrow. DNA and protein adducts in liver reached maximum levels 30 minutes after benzene administration, whereas those in bone marrow reached maximum levels after six hours. Both protein and DNA adduct AUCs were greater in bone marrow than in liver. Dose-response assessments at both 1 and 12 hours showed that DNA and protein adducts in liver and bone marrow were dose dependent over doses spanning eight orders of magnitude. Consistent with the benzene metabolism data, these data show that reactive forms of benzene were present in liver and bone marrow after exposure to human-relevant benzene levels. Thus, at low doses, benzene was absorbed and metabolized into reactive intermediates capable of binding to DNA and protein.

The relation between benzene metabolism and macromolecular binding was examined by comparing benzene macromolecular adduct formation among strains of male mice (B6C3F$_1$, DBA/2, and C57BL/6) and male rats. These animals have been reported to have different metabolic capacities for benzene and also different tumorigenic and cytotoxic responses to benzene exposure. We hypothesized that differences in the capacity to metabolize benzene affect macromolecular adduct formation and that the amount of macromolecular damage is related to benzene's ability to cause cancer and other blood disorders. ^{14}C-benzene was administered intraperitoneally to all rodents (5 µg/kg body weight) and adduct levels were determined by AMS at selected time points up to 48 hours after exposure. AUCs for protein and DNA adducts in bone marrow, the primary target organ for benzene toxicity, decreased in the following order: B6C3F$_1$, DBA/2, C57BL/6, and rats. Similarly, adduct AUCs in liver were greater in B6C3F$_1$ mice than in rats although the trend was less clear for the DBA/2 and C57BL/6 mouse strains. The results of this work are consistent with previously published work showing

* A list of abbreviations and other terms appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute Research Report 113, which also includes a Commentary by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Kenneth W Turteltaub, BBR–Program, 7000 East Avenue, PO Box 808, L-452, Livermore CA 94554.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R82811201 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

that the ability to metabolize benzene follows a similar pattern with these animals and is consistent with the tumorigenicity of benzene in mice and rats. Thus, our data suggest that benzene toxicity is related to the ability to produce macromolecular adducts.

Preliminary studies were conducted to assess adduct dosimetry after low-dose inhalation of benzene. Inhalation methods were developed to administer a nominal body burden of 5 µg benzene/kg body weight. Then, ^{14}C-benzene was administered to B6C3F_1 mice and rats by both intraperitoneal (IP) administration and by inhalation, and DNA and protein adducts in liver and bone marrow were analyzed by AMS. AUCs for adduct levels were greater after IP benzene administration than after inhalation of benzene. Adduct levels were greater in DNA from B6C3F_1 mouse bone marrow than in DNA from liver regardless of exposure route. Collectively, these data show that the internally reactive dose was greater when benzene exposure was by IP administration.

In summary, the results suggest that benzene is metabolized to reactive forms capable of binding both protein and DNA in target and nontarget organs of rats and mice at doses encountered by humans through environmental exposure. Macromolecular binding was dose-dependent at low doses of benzene and reflected benzene toxicity, based on its carcinogenicity and ability to cause other blood-related disorders. These data are consistent with macromolecular adducts being indicative of benzene exposure and benzene toxicity although much more research is needed to validate this point. Additionally, benzene metabolism varies among species and among strains within a species of rodent. Thus data are needed in humans to understand how to use the rodent data in risk assessment and ultimately to determine whether macromolecular adducts are a useful indicator of exposure and a useful predictor of risk.

INTRODUCTION

Benzene is a well-known industrial chemical and pollutant that is distributed widely in the environment via fossil fuel combustion, motor vehicle exhaust, forest fires, and other combustion processes. Other common sources of exposure include tobacco smoke and volatilization during the fueling of automobiles (Wallace et al 1984; Wallace 1989). Benzene is also used industrially as a solvent and as a raw material for chemical synthesis.

A variety of studies have been conducted to estimate human exposure to benzene from environmental and occupational sources (Wallace 1984; Smith 1996; Wallace 1996; Cocheo et al 2000). Major environmental sources of exposure are active and passive smoking, auto exhaust, and driving or riding in automobiles. These studies indicate that benzene concentrations in outdoor air range from less than 5 µg/m^3 to 20 µg/m^3, depending on location and time of day. Personal exposures, based on sampling of the air microenvironments surrounding study participants, are estimated to range between 6 and 29 µg/m^3. Exposure via indoor air is estimated to be 2 to 8 µg/m^3, and exposure while driving automobiles averages about 10 to 40 µg/m^3. After active smoking, some of the highest exposures occur while filling automobile gasoline tanks during which inhalation doses are estimated at 80 to 100 µg/person per tank-filled. Body burden estimates from ambient exposures, based on benzene concentration in blood measured in 900 randomly selected people, are 15 to 1800 ng/L blood, with a mean of 131 ng/L. Wallace and colleagues (1984) suggest that the average person living in an urban environment inhales 0.6 mg benzene a day.

Filling station attendants reportedly are exposed to approximately 0.53 mg/m^3 benzene per year, with benzene/air concentrations of 0.003 to 0.6 ppm in the immediate microenvironment (Carere et al 1995; Nillson et al 1996). Mean exposures for benzene plant and cokery workers have been reported to be 0.9 cm^3/m^3 time-weighted average (2.9 mg/m^3); and the highest individual value was 15 cm^3/m^3 time-weighted average (49 mg/m^3) (Kivistö et al 1997). Reported occupational exposures to benzene in 30 other job categories range between 3 and 68.7 mg/m^3 with air concentrations of 0.003 to 8.20 ppm (Tompa et al 1994).

Thus, ambient human exposure to benzene can range widely depending on location and activity but is generally within 2 to 30 µg/m^3 with body burdens of a few hundred nanograms per liter blood per person. People exposed occupationally to benzene may receive exposures 100 to 1000 times greater than ambient exposures. Smoking, which provides the greatest environmental exposure to benzene, results in body burdens 5 to 10 times those of nonsmokers.

Exposure to benzene is a health concern. Benzene is a multiorgan carcinogen in mice (Snyder and Kocsis 1975; Maltoni et al 1983; Huff et al 1989; Maltoni et al 1989; Snyder and Kalf 1994). In humans, exposure to high levels of benzene, such as those in occupational settings, leads to blood disorders in humans such as pancytopenia (Goldstein 1977) and leukemia (Aksoy and Erdem 1978; Cronkite et al 1984). Regulations limit the maximum permissible occupational benzene exposure to 1 ppm in air averaged over eight hours of work with a 40-hour work week (US Occupational Health and Safety Administration 1987; Rinsky et al 1989; American Conference of Governmental Industrial Hygienists 1999).

Because benzene exposure is unavoidable and presents health risks at occupational doses, understanding what risks benzene may present at the lower doses people receive in the environment is important. Molecular markers of benzene-induced biological damage would help determine this risk. Benzene toxicity (indicated by blood disorders and cancer) is generally believed, although not proven, to result from metabolism of benzene to reactive intermediates (see Figure 1 for pathways) that then interact with DNA or protein. Large differences have been found in benzene toxicity among various animal models (US Department of Health and Human Services 1986; Sabourin et al 1988a, 1992), and these differences may be due to differences in the ability to metabolize benzene (metabolic capacity). Differences among humans in benzene metabolism correlate with incidence of nonlymphocytic leukemia and related myelodysplastic syndromes (Rothman et al 1997). Several studies have suggested that the dihydroxylated metabolites of benzene (hydroquinone and catechol), as well as muconaldehyde, are toxic (Snyder and Kalf 1994; Snyder and Hedli 1996). These metabolites may lead to formation of macromolecular adducts, which represent the amount of carcinogen that has become metabolically activated and subsequently has bound covalently to DNA and protein. Benzene may also stimulate production of reactive oxygen or nitrogen species that, both alone and in combination with specific benzene metabolites, damage DNA and protein (Eastmond et al 1987; Laskin et al 1987; Kolachana et al 1993; Tuo et al 1999).

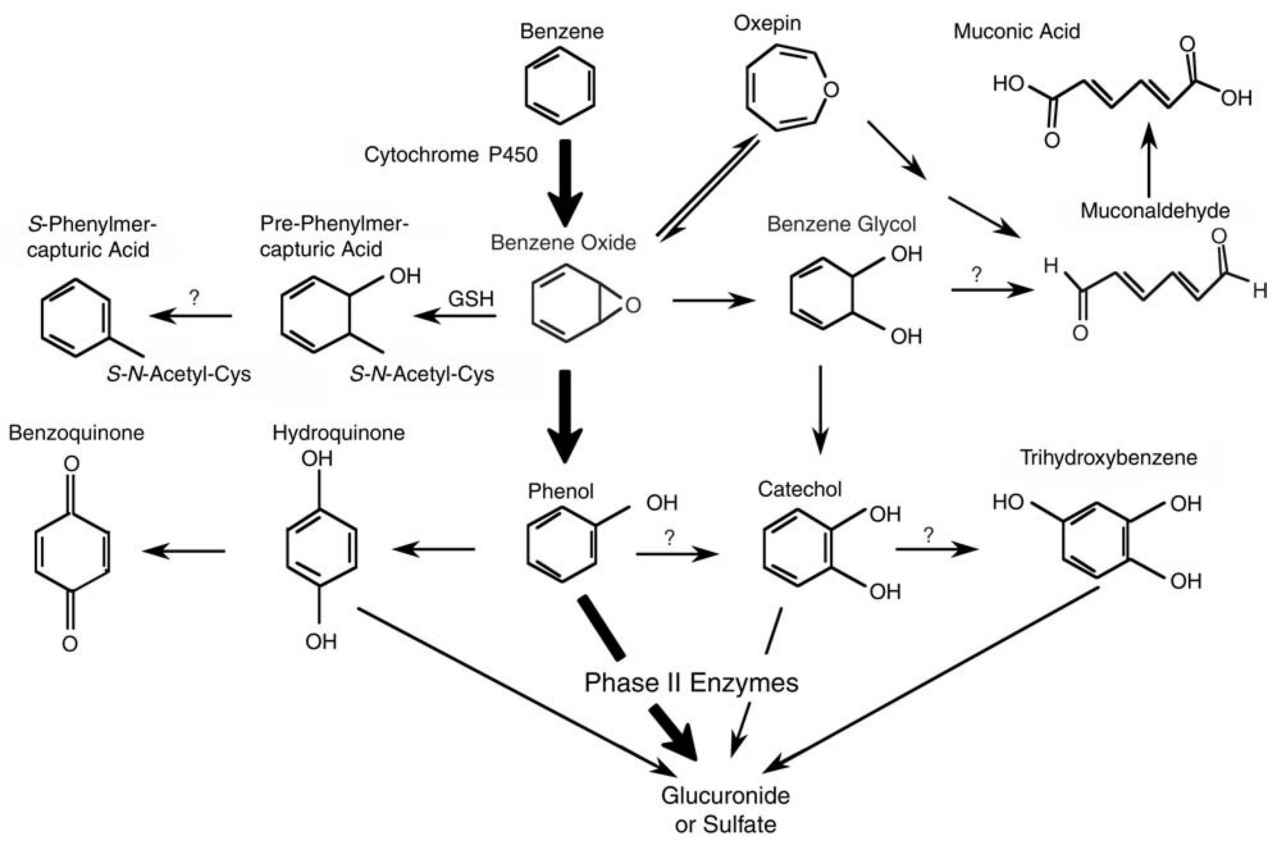

Figure 1. Important pathways of benzene metabolism in rodents. Most benzene is oxidized to phenol and conjugated to glucuronide or sulfates (this pathway is indicated by bold arrows). Pathways leading to the formation of muconaldehyde and phenylmercapturic acid are also shown.

Thus, we hypothesize that differences in the metabolism of benzene and in the resulting levels of macromolecular adducts will indicate the potential for overt toxicity such as development of cancer. Importantly for this hypothesis, both metabolites and macromolecular adducts can be quantitatively measured and characterized prior to development of tumors and thus may be early indicators of both exposure and risk. Studying these endpoints after low-dose benzene exposure should help determine whether benzene can present a health risk at its current environmental exposure levels and whether its metabolites and adducts can be used to estimate exposure or risk in humans.

At present, very little is known about the health risks posed by benzene in urban air (Cronkite 1987; Cronkite et al 1989; Huff et al 1989; Sabourin et al 1989; Snyder et al 1993). DNA adducts have been found in rodents after administration of benzene (Snyder et al 1978; Arfellini et al 1985; Bauer et al 1989; Mazzullo et al 1989; Bodell et al 1994; Pathak et al 1995; Levay et al 1996; Creek et al 1997), and one benzene DNA adduct has been characterized (Pongracz and Bodell 1996). Benzene has also been shown to form protein adducts in tissues and adducts with albumin and hemoglobin in blood (Mazullo et al 1989; Sun et al 1990; Creek et al 1997; Yeowell-O'Connell et al 1998). However, the relations among metabolism, adduct formation, and susceptibility, especially at low doses of benzene, are currently unknown.

Conducting such low-dose studies has historically been difficult due to limitations in the available analytic methods. We believe, however, that such studies are possible using radioisotope-labeled benzene and the ultrasensitive AMS. This method for analysis of isotope ratios can measure ^{14}C into the low attomole (10^{-18} mol) range with precision of greater than 5% (Turteltaub et al 1993; Turteltaub and Vogel 1995; Vogel et al 1995).

SPECIFIC AIMS

The purpose of this study was to explore the use of AMS to assess benzene dosimetry in rodents after doses relevant to human ambient exposure. We also intended to study whether metabolite or adduct levels can be used to distinguish between exposure groups and animal models with different biological responses to benzene challenge. This work assumed that benzene toxicity requires metabolism to reactive intermediates that bind macromolecules and that differences in adduct levels represent differences in the kinetics and metabolism of benzene.

The specific objectives were to:

1. Determine how dose influences benzene metabolism by measuring metabolite profiles in male B6C3F$_1$ mice after administering IP benzene (5 ng/kg to 500 mg/kg body weight), placing particular emphasis on the metabolites that may be markers of benzene bioactivation (hydroquinone, muconic acid, catechol, and phenol).
2. Investigate how benzene metabolism leads to macromolecule damage at doses directly relevant to humans by comparing metabolite profiles with macromolecular adduct levels in bone marrow of male B6C3F$_1$ mice.
3. Compare the formation of benzene macromolecular adducts among different types of rodents (three mouse strains and rat) after exposure to low doses of benzene.
4. Begin developing methods for conducting low-dose inhalation studies in combination with AMS.

METHODS AND STUDY DESIGN

CHEMICALS AND METABOLITE STANDARDS

[U-^{14}C]-benzene (58.3 mCi/mmol; 99% radiopure) was obtained from Sigma Chemical Company (St Louis MO). Radiopurity was determined by high-performance liquid chromatography (HPLC; model 1084B, Hewlett-Packard, Palo Alto CA) equipped with a C-18 column (10 mm internal diameter × 25 cm; Rainin, Emeryville CA). The analysis was carried out isocratically (70% [vol/vol] methanol/30% [vol/vol] water) at a flow rate of 1 mL/min. Metabolites were detected by ultraviolet (UV) absorption at 254 nm and liquid scintillation counting of fractions collected at one-minute intervals. Under these conditions, benzene eluted at approximately seven minutes and no contaminant peaks were observed.

Benzene (used to dilute the specific activity of ^{14}C-benzene) was obtained from Aldrich Chemical Company (Milwaukee WI). Ribonuclease (RNase) A (enzyme classification number [EC] 3.1.27.5), RNase T1 (EC 3.1.27.3), proteinase K (EC 3.4.21.14), 3-(*N*-morpholino)propanesulfonic acid (MOPS), urea, and Triton-X 100 were obtained from Sigma. Tributyrin was obtained from ICN Biochemicals (Cleveland OH). Protein assay reagent and dithiothreitol (DTT) were obtained from Pierce (Rockford IL). All other chemicals were analytic grade.

Catechol, hydroquinone, muconic acid, and phenol, all greater than 99% pure, were obtained from Sigma. Hydroquinone sulfate, phenyl glucuronide, and phenyl sulfate were obtained from Dr William Bechtold at the Lovelace Respiratory Research Institute (Albuquerque NM).

ANIMALS

Male B6C3F$_1$, DBA/2, and C57BL/6 mice (30 g) and male Fischer rats (200 g; F344/Sim) were from Simonsen Laboratories (Gilroy CA). These animals were selected because of their previous use in tumor and genotoxicity studies and because published data suggest that they differ in susceptibility to benzene toxicity. Prior to the study, all protocols utilizing animals were reviewed and approved by the Institutional Animal Care and Use Committees at the Lawrence Livermore National Laboratory and the Lovelace Respiratory Research Institute. Before use, animals were acclimatized for a minimum of one week in an animal care facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Animals were housed, three per cage, in filter-top, polycarbonate cages with hardwood-chip bedding. They were given water and food ad libitum, kept on a 12-hour light/dark cycle, and maintained at approximately 22°C throughout the study.

BENZENE ADMINISTRATION

Two different routes of exposure were used in this project: IP and inhalation. All dose-response studies were carried out by IP administration. The toxicokinetics studies were carried out using both the IP and inhalation routes.

IP Administration

For dose-response studies, appropriate doses (see Figures 3, 4, and 6) were obtained by diluting the specific activity of radiolabeled benzene with unlabeled benzene so that each animal received 0.1 µCi of ^{14}C-benzene in corn oil (200 µL/animal). Control animals were given corn oil only. For the toxicokinetic studies using the IP route, animals were treated with 5 µg ^{14}C-benzene per kg body weight in corn oil (200 µL/animal) and killed by carbon dioxide (CO_2) asphyxiation at intervals from 0 to 48 hours afterward (see Tables 5 and 6 for time points). Dosing solutions were used within two weeks of preparation.

Inhalation Exposure: Rats

Inhalation exposure of rats was carried out at the Lovelace Respiratory Research Institute using methods reported by Sabourin and colleagues (1987) under the supervision of Dr Janet Benson. Briefly, a J-tube vapor generator was used to produce ^{14}C-benzene vapor. The benzene exposure concentration was continuously monitored by infrared spectroscopy (Foxboro Miran, Wilkes Scientific, Norwalk CT).

To determine the toxicokinetics of benzene inhalation exposure, rats were exposed by nose only to 0.1 ppm ^{14}C-benzene (0.09 µCi/animal), delivered at a rate of 0.2 L/min for 20 minutes, which resulted in a nominal delivered dose of 5 µg/kg body weight (see calculations below).

The concentration of benzene in air was determined using the following general formula:

$$\text{mg/m}^3 = [\text{ppm}] \times [\text{Molecular Weight}/22.4] \times [P/\text{P}_0] \times [\text{T}_0/T](1) \quad (1)$$

Thus, assuming a barometric pressure (P) of 620 mm Hg (Albuquerque NM) and an exposure chamber temperature (T) of 293 kelvin (20°C) while T_0 is 273 kelvin (0°C) and P_0 is partial pressure of 760 mm Hg, the equation becomes

$$\begin{aligned}\text{mg/m}^3 &= \mu\text{g/L}\\ &= 0.1 \times [78/22.4] \times [620/760] \times [273/293]\\ &= 0.26\ \mu\text{g/L Benzene in Air.}\end{aligned}$$

The delivered benzene dose was then determined using formula (2):

$$\begin{aligned}\text{Body Burden} &= 0.26\ \mu\text{g/L} \times 0.2\ \text{L/min}\\ &\quad \times 20\ \text{min/rat} \qquad (2)\\ &= 0.0052\ \mu\text{g/rat}\\ &= 5\ \mu\text{g/kg for a 200g rat.}\end{aligned}$$

After exposure, the rats were housed in polycarbonate chambers in a negative airflow unit. At 0, 0.5, 1, 3, 12 and 24 hours after the end of the exposure, three animals per time point were killed by CO_2 asphyxiation and their tissues collected for analysis by AMS.

Inhalation Exposure: Mice

The inhalation chamber at the Lovelace Respiratory Research Institute was not equipped to deliver the 5 µg/kg dose of benzene by nose-only exposure to mice due to the low body weight. Therefore, an inhalation chamber was constructed in Livermore, California, for this purpose: using a 4-L glass jar, 3 holes were made in the lid for inserting mice. Each hole was fitted with a 50-mL centrifuge tube with another hole cut at the bottom large enough to restrain one mouse for nose-only inhalation. This setup positioned the nose a few millimeters into the jar. A fourth hole in the lid housed a small injection port into which a gas-tight syringe was inserted for delivery of ^{14}C-benzene and for sampling the benzene concentration in the air.

To facilitate benzene evaporation, the inhalation chamber was heated to 50°C from the bottom with an infrared lamp. The lamp was turned off just before benzene injection: 40 µL of a 0.07% ^{14}C-benzene solution in water was injected into the chamber and allowed to equilibrate for two minutes. Preliminary studies showed that an average of 50% (30% to 70%) of the benzene volatilized, giving a nominal benzene concentration of 0.1 ppm in the chamber at standard temperature and pressure (STP) using this method. The volatilized fraction was constant for up to six minutes. This was verified by sampling the chamber air with a gas-tight syringe and analyzing its contents by liquid scintillation counting after bubbling into ethanol. The concentration of the benzene placed in the glass jar was adjusted to correct for the volatilized fraction. Animals were exposed to 0.1 ppm benzene in air for 2.5 minutes, which attained a nominal body burden for benzene equivalent to 5 µg/kg body weight, assuming a 30 g mouse and a minute volume of 0.04 L.

The concentration of benzene in air was determined as described in equations (1) and (2), assuming a barometric pressure of 760 mm Hg (Livermore CA) and an exposure chamber temperature of 293 kelvin (20°C):

From equation (1):

$$\begin{aligned} \text{mg/m}^3 &= \mu\text{g/L} \\ &= 0.1 \text{ ppm} \times [78/22.4] \\ &\quad \times [760/760] \times [273/293] \\ &= 1.6\ \mu\text{g/L Benzene in Air.} \end{aligned}$$

From equation (2):

$$\begin{aligned} \text{Body Burden} &= 1.6\ \mu\text{g/L} \times 0.04 \text{ L/min} \times 2.5 \text{ min/mouse} \\ &= 0.006\ \mu\text{g/mouse} \\ &= 5\ \mu\text{g/kg for a 30g mouse.} \end{aligned}$$

After the inhalation procedure, animals were returned to their cages and were kept in negative airflow units. At specific time points after the exposure (see Figures 8 and 9, Tables 5 and 6), animals were killed by CO_2 asphyxiation and their tissues collected for analysis.

For these time-course studies, historical data on the ^{14}C content in unexposed animals was used as background. The natural amount of radiocarbon in all biological material is constant in these animals due to their age and growth conditions and generally varies by less than 10%.

SAMPLE COLLECTION AND HPLC ANALYSIS

Plasma and Tissue Metabolites

Blood was drawn by cardiac puncture and placed into vials containing 25 units of heparin. Plasma was separated from whole blood by centrifugation at 3000*g* for 10 minutes. The plasma was removed, frozen in liquid nitrogen, and stored at −35°C until metabolite analysis. Liver was immediately placed on dry ice and stored at −35°C until use. Bone marrow was collected by flushing each femur and humerus with approximately 1 mL of phosphate-buffered saline (PBS), pH 7.4, as previously reported (Creek et al 1997). Tissues were usually stored for less than two weeks.

For extraction of metabolites, plasma (100 µL), liver (100 mg) and bone marrow from one femur and one humerus were homogenized using a handheld, motorized homogenizer in 0.5 mL of buffer (0.1 M sodium ascorbate, 25 mM sodium phosphate, 30 mM (+)D-saccharic acid-1,4-lactone; pH 7.0). PNPG (*p*-nitrophenyl glucuronide) was used as an internal standard to determine extraction efficiency. Each homogenate was transferred to disposable glass tubes and 3.5 mL of ethyl acetate containing benzene metabolite standards was added (Sabourin et al 1988a). The ethyl acetate standard solution contained 1.3 mL benzene, 85 mg phenol, 63 mg catechol, 630 mg hydroquinone, and an antioxidant (10 g butylated hydroxytoluene) in 500 mL of ethyl acetate purged with nitrogen. Each homogenate plus ethyl acetate was centrifuged for 15 minutes at 3000*g* to separate the aqueous and organic phases.

Each aqueous layer was extracted twice with one volume of cold ethyl acetate, and the ethyl acetate fractions were pooled. An equal volume of methanol was added to the aqueous solution and the solution was kept on ice for 20 minutes to precipitate protein. The precipitate was pelleted by centrifugation at 3000*g* for 15 minutes. To remove methanol, which interferes with HPLC analysis, each aqueous fraction was then evaporated under nitrogen to one half its volume.

Individual metabolites were separated and quantitated by HPLC (as reported by Sabourin et al 1988a). Data were normalized to the recoveries of the internal standard and were expressed as femtomoles of [^{14}C]-labeled metabolite per milliliter HPLC eluent (Bechtold et al 1988). Ethyl-acetate tissue extracts were analyzed using a Rainin HPLC system equipped with a C-18 phenomenex-DB semipreparatory column (250 × 10 mm, pore size 8 µm). Mobile phases were acetonitrile (hereafter called solvent A) and 10 mM KH_2PO_4 buffer (hereafter called solvent B) with uncorrected pHs. Samples were injected after the column was equilibrated at 100% solvent A. The composition of the solvents was changed using a linear gradient from 0% to 10% solvent B over 5 minutes. From 5 to 30 minutes, the solvent composition was modified from 10% solvent B to 100% solvent B and then held isocratic for an additional 10 minutes. Metabolite detection and quantification was carried out as described for urinary metabolites below.

Urinary Metabolites

Urine was collected for 24 hours after exposure into vials containing 2% ascorbic acid to prevent oxidation of metabolites. These samples were stored in the dark at −35°C until analyzed. Urine samples were each centrifuged at 600*g* for 15 minutes to remove particulates. Urinary benzene metabolites were then analyzed by HPLC as follows: A maximum of 10 disintegrations per minute of [^{14}C]-containing urine was injected onto an Econosphere C18 HPLC column (10-µm particle size, 250 mm length, 4.6 mm internal diameter; Alltech, Deerfield IL) equipped with a Brownlee C-18 guard cartridge (10-µm particle size). The metabolites were separated using a Rainin binary system with solvents A and B as described in Sabourin and colleagues (1988b). Two different concentrations of tetrabutylammonium hydrogen sulfate (TBAHS) solution, 50 mM TBAHS in deionized water and 35 mM TBAHS in methanol, were prepared. Solvent A contained 90% aqueous TBAHS and 10% methanolic TBAHS; solvent B contained 20% aqueous TBAHS and 80% methanolic TBAHS. Separation was carried out with a linear gradient starting at 0% solvent B for five minutes to 50% solvent B over 30 minutes, with a flow rate of 1 mL/minute. Metabolites were detected by UV absorption at 265 nm using a 1050 diode array detector (Hewlett-Packard). HPLC fractions were collected at one-minute intervals and 300-µL aliquots of each fraction were dried in a vacuum centrifuge and prepared for AMS analysis using previously published methods (Turteltaub et al 1993).

ISOLATION OF DNA FROM TISSUE SAMPLES

DNA was isolated as previously reported (Creek et al 1997). Briefly, approximately 100 mg of pulverized liver or bone marrow from one femur and one humerus were solubilized overnight in 4 to 5 mL freshly made lysis buffer containing 4 M urea, 10 mM Tris-HCl (pH 8.0), 1% Triton-X100, 10 mM DTT, 100 mM NaCl, and 800 µg/mL proteinase K at 37°C in a shaking water bath. Samples were then centrifuged to remove undigested tissue. RNase A (0.5 mg/mL final concentration) and RNase T1 (2500 units final concentration) were added to the supernatant and incubated for 30 to 60 minutes at 37°C to digest RNA. The samples were then loaded onto anion exchange columns (QIAGEN 500, QIAGEN, Chattsworth CA).The columns had been preequilibrated with loading buffer (750 mM NaCl, 50 mM MOPS, 15% ethyl alcohol [EtOH],and 0.15% Triton X100; pH 7.0), washed three times with washing buffer (1 M NaCl, 50 mM MOPS, and 15% EtOH; pH 7.0), and eluted in elution buffer (125 mM NaCl, 50 mM MOPS, and 15% EtOH; pH 8.0). To precipitate DNA, samples were mixed with one volume ice-cold isopropyl alcohol, were stored for at least 12 hours at −20°C, and were centrifuged at 3000 rpm for 3 to 4 hours at 4°C. This procedure yielded about 100 µg of DNA with an A260/A280 ratio of 1.7 to 1.9 per 100 mg tissue. DNA concentrations were determined by UV absorption at 260 nm, assuming that an A260 of 1.0 represents 50 µg/mL DNA.

ISOLATION OF PROTEIN FROM TISSUE SAMPLES

Approximately 100 mg liver or bone marrow from one femur and one humerus was pulverized and lysed with the procedure used for DNA isolation without the added proteinase K. After centrifugation, all unsolubilized tissue was removed by decanting the supernatant. Perchloric acid (PCA; 70% vol/vol) was added to the supernatant to give a final concentration of 5% PCA. The samples were centrifuged at 1800*g* for 15 minutes at room temperature, and the pellet was resuspended in 1 to 2 mL of ice-cold 5% PCA. The precipitate was then pelleted by centrifugation at 1800*g* and washed twice with 50% (vol/vol) methanol and once with 50% (vol/vol) ethanol/ether. The resulting pellet was allowed to air dry and was resuspended in 0.1 M potassium hydroxide (KOH). The protein content of the extract was determined using the Bradford microassay (Pierce Co, Rockford IL) according to the manufacturer's instructions and with bovine serum albumin as the standard.

AMS ANALYSIS

AMS was used to determine the radiocarbon content of samples, DNA extracts, protein extracts, and metabolite fractions. Protein isolates and HPLC fractions from the urine metabolite analysis were prepared. Two milligrams of a carbon carrier, tributyrin, was added to each DNA extract and HPLC fraction from liver, plasma and bone marrow in order to provide the necessary carbon content for efficient sample handling. Each sample was placed in a clean quartz tube (6 mm × 30 mm, 4 mm internal diameter) that had been previously baked at 600°C. To avoid contamination through handling, exchange of volatiles, and aerosols, each quartz tube was placed inside the inner of two nested borosilicate glass culture tubes (10 mm × 75 mm and 12 mm × 100 mm) and covered with glass fiber filter paper (GF/A, 21 mm, Whatman, Springfield Mill, Kent, UK). Samples in the tubes were then dried in a vacuum centrifuge. The borosilicate tubes containing samples were handled only with gloves.

The dried samples were then converted to graphite using a two-step process (Vogel 1992). Briefly, the samples in the quartz tubes were removed from the borosilicate tubes with clean forceps and placed into new quartz tubes (6 mm × 50 mm), evacuated, and oxidized to CO_2 by heating at 900°C for four hours in the presence of copper oxide. The CO_2 was then cryogenically transferred to a second 6 × 50 mm quartz tube under vacuum, using a Nalgene "Y" connector, and was reduced to filamentous graphite in the presence of cobalt at 900°C. The resulting graphite on cobalt was placed in the ion source of the spectrometer and analyzed using previously published protocols (Turteltaub et al 1993).

AMS measures the ratio of a rare isotope relative to a stable isotope. For ^{14}C-benzene analysis, we measured the concentrations of ^{14}C relative to ^{13}C and normalized to the $^{14}C/^{12}C$ ratio of 1950 carbon using the Australian National University sugar reference standard (Vogel et al 1995). The ratios are converted to mass of ^{14}C-benzene based on the specific activity of the benzene after subtracting the natural radiocarbon contributed from the sample and tributyrin carrier (when added). Carbon content of the samples was determined using a C/N/S analyzer (Carlo-Erba NA1500, series 2, CE Elantech, Lakewood NJ). According to this analysis, tributyrin, DNA, and protein contained 59.6%, 29%, and 31% carbon, respectively. Radiocarbon is a cosmogenic isotope that is present in all biological material. Present-day natural levels (background) of ^{14}C in living biological materials is 112 ± 5 amol/g carbon. Tributyrin, based on analysis of over 500 samples, contains 15 ± 2 amol ^{14}C/mg tributryin. AMS measures radiocarbon content only and gives no information on the origin or source of the radiocarbon. All radiocarbon data are therefore collectively termed *benzene equivalents*.

All samples were counted to 10,000 total counts (1% counting error). Each sample was also analyzed up to seven times or until the measurement coefficient of variation of the intrasample measurement was within 5%, whichever came first. In no case did the intrasample measurement variability exceed 5%. Up to three replicate animals were used at each time point. The mean radiocarbon content of tributyrin, which was used to increase the carbon content of some samples, was determined by preparing and analyzing at least three independent samples per set of experimental samples.

STATISTICAL METHODS AND DATA ANALYSIS

All analyses were performed using S-PLUS 2000 (Data Analysis Products Division, Mathsoft, Seattle WA).

Dose-Response Analyses

The logarithm of the response was used as the dependent variable. Taking logarithms stabilized the variance across the several orders of magnitude in response from low to high doses. Also, due to the large range of doses considered, the logarithm of dose was used as the independent variable, rather than the dose itself.

For dose response curves for plasma, liver, and marrow and four metabolites (catechol, hydroquinone, muconic acid, and phenol), only three distinct doses were available. Consequently, we ran simple linear regressions of the logarithm of response on the logarithm of dose. Confidence intervals for parameters were simple point-wise confidence intervals. That is, we did not adjust for multiple testing in this case because a prescribed set of tests was not specified in advance.

AUC Calculations

In all cases, integrals of estimated functions of response versus hours after exposure were themselves estimated by:

1. using generalized least squares (GLS) to estimate the average response at each time point where there were data,
2. using error estimates per time point from the GLS fit to estimate the precision of each estimated response,
3. using the trapezoidal rule to estimate the integral, and
4. combining the error estimates per time point to estimate the standard error in the trapezoidal rule estimate.

GLS (rather than ordinary least squares) was used because the observed variation in error at each time point was great. GLS procedures permit more precise fits in situations like these, as long as a parametric relation between the mean and variance can be assumed. Because the log-transformed data showed approximately constant variance within each set of time-course data, the nontransformed data should have a variance that, to first order, would be proportional to the mean. In addition, for these experiments, only a small number of animals (two or three) were used at each time point. Hence, standard error estimates for the mean value per time point based on the empirical standard deviation of the two or three data points would be extremely unreliable. The only way to obtain reliable estimates of standard errors for average concentrations at each time point, which are needed to obtain standard errors for the AUCs, was to make reasonable assumptions about the relation between mean and variance and to use GLS to estimate parameters of the mean-variance relation. For these data, the variance was assumed to be proportional to a power of the mean, and the exponent and constant of proportionality were estimated along with the means. We used the S-PLUS GLS program for estimation (Pinheiro and Bates 2000).

The trapezoidal rule for estimating an AUC, along with procedures for estimating its standard error, are fully described in reports by Bailer (1988) and Bailer and Piegorsch

(1990). Generally, suppose we want to estimate the area under an unknown concentration function $f(t)$ constrained such that $f(0) = 0$. We have a set of n time (t)-concentration (c) pairs $(t_1, c_1), (t_2, c_2), \ldots, (t_n, c_n)$, where $0 < t_1 < t_2 < \ldots < t_n$, and c_i is measured with variance (v) v_i. The trapezoidal rule defines a simple set of weights (w) $w_1, w_2, \ldots, w_n$; the area under f from 0 to t_n can be estimated as $w_1c_1 + \ldots + w_nc_n$. The variance of that estimate is given by $w_1^2v_1 + \ldots + w_n^2v_n$.

For the species comparison only, data were available that allowed calculation of a possible background effect or bias due to ^{14}C from sources other than the labeled benzene. These AUC estimates were corrected for background effect by subtracting the minimum observed value in a data series from each point in the series and then applying the algorithm to these adjusted data. (Whenever the minimum observed value in a series was less than zero, no adjustment was performed.) The adjustment was carried out to evaluate the effects of possible biases introduced by sample preparation or AMS measurement. For these experiments, sample preparation and measurement were not randomized. Rather, samples for the rat or each strain of mouse were prepared at the same time and measured consecutively. Hence, systematic differences between data series could be due in part to biases introduced during these processes. Estimating the AUC that is above a maximal positive baseline provides a simple alternate statistic that can be used in comparisons. Both the unadjusted and adjusted AUCs are presented in the tables.

Multicomparison Analyses

The AUCs for the IP experiments were calculated out to 48 hours. Inhalation data, however, were available only out to 24 hours. Consequently, the IP AUCs were also calculated only out to 24 hours before comparison to inhalation AUCs. The asymptotic confidence intervals for the difference between inhalation and IP AUCs were adjusted to account for the eight comparisons (two tissues × two adducts × two species) in the inhalation versus IP analyses. The adjustment method used was Sidak's method, as described in the *S-PLUS User's Guide* (Mathsoft 1999).

Z tests were used to test for differences among estimated AUCs for bone marrow DNA adducts, as well as for differences among estimated AUCs for liver macromolecular adducts. The standard error of the difference between two means was estimated as the square root of the sum of the squared standard errors for the two means being compared. Test significance thresholds were also adjusted for multiple comparisons using the Sidak method.

RESULTS

BENZENE METABOLISM AT LOW DOSES IN B6C3F$_1$ MICE

Metabolite Analysis by HPLC

An example of the distribution of radiocarbon across an HPLC separation of urine from a B6C3F$_1$ mouse is shown in Figure 2. The radiocarbon levels in the HPLC fractions collected at one-minute intervals are shown as a histogram, and the UV absorption of the fractions at 265 nm is shown as a continuous trace (gray). The UV trace indicates the retention time for the authentic metabolite internal standards, which were added into the urine samples before HPLC separation. The radiocarbon profile corresponded well with retention of the authentic standards although one major radiolabeled peak eluting at eight minutes did not correspond to any standards and its identity is presently unknown. All the major metabolites of benzene are well resolved and detectable to levels as low as a few attograms benzene equivalents per milliliter urine, assuming the limit of detection was a signal-to-noise ratio of three.

Dose-Dependence of IP Benzene Metabolism

Benzene metabolites in urine of B6C3F$_1$ mice were quantified by HPLC-AMS after administering doses of 50 ng/kg to 500 mg/kg body weight. Because metabolites were difficult to identify and quantify below a dose of

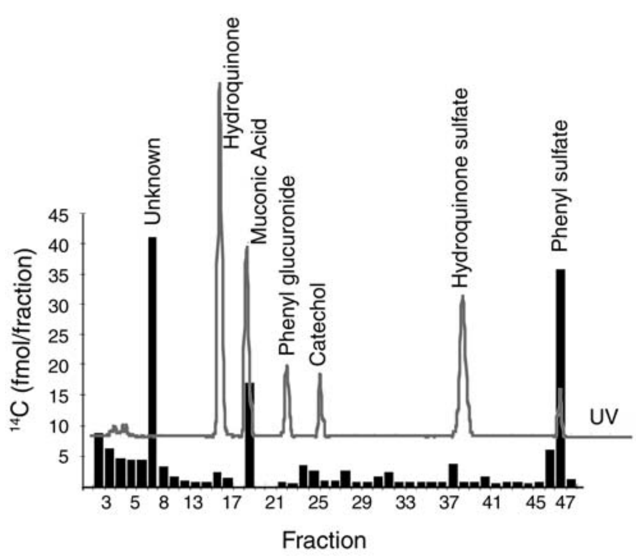

Figure 2. HPLC-AMS chromatogram of urine collected over 24 hours from a B6C3F$_1$ mouse administered 5 µg ^{14}C-benzene per kg body weight. Phenol (not shown) eluted in fraction 33 (at 33.3 minutes) using this HPLC system. The solid line represents the UV trace of standards (labeled by name). Bars represent the radiocarbon content of each HPLC fraction.

5 ng/kg body weight, all dose-response studies using urine were conducted using doses above 5 ng/kg body weight. Three major radiocarbon peaks were detected at all doses analyzed: phenyl sulfate, muconic acid, and the uncharacterized peak described earlier. In addition, two minor metabolites corresponding to phenyl glucuronide and hydroquinone sulfate were observed at all doses (Figure 3). Free catechol and free hydroquinone were also detected at low levels but were not dose dependent (shown in Appendix A). Radioisotope levels in urine corresponding to the elution of phenol were very low and were detected above background only at the highest doses.

Although the amounts of the three major metabolites detected (phenyl sulfate, muconic acid, and the uncharacterized peak) were dose dependent, their responses tended to follow an S-shape with the curves flattening at higher doses. The detected amount of each metabolite relative to one another remained in the same order over the entire dose range with the unidentified metabolite ≥ phenyl sulfate > muconic acid ≥ phenyl glucuronide > hydroquinone sulfate. These relations are based on the average value of each data point at the lower asymptote.

Figure 4 illustrates the dose response for benzene metabolites in liver, bone marrow, and plasma one hour after exposure to doses of 5 ng, 50 ng, and 5 µg benzene per kg body weight. Because of their postulated role in benzene toxicity, only muconic acid, hydroquinone, catechol, and phenol were analyzed in these samples. The best linear fits for the log-transformed dose-response data are shown. In

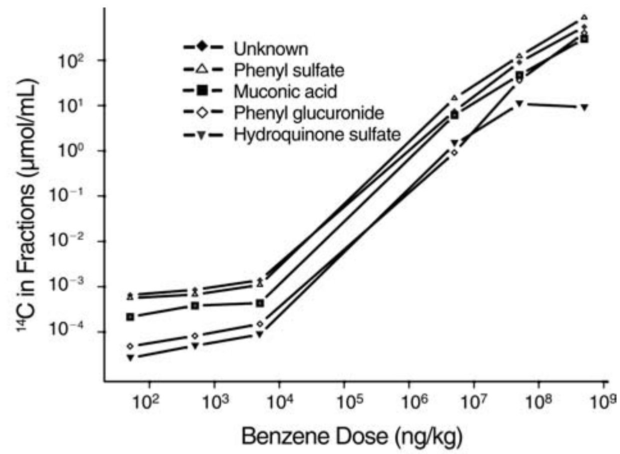

Figure 3. Dose response for five metabolites in urine from B6C3F$_1$ mice. Geometric means of ^{14}C in HPLC fractions are plotted against administered dose. Curves were generated by interpolating between the means.

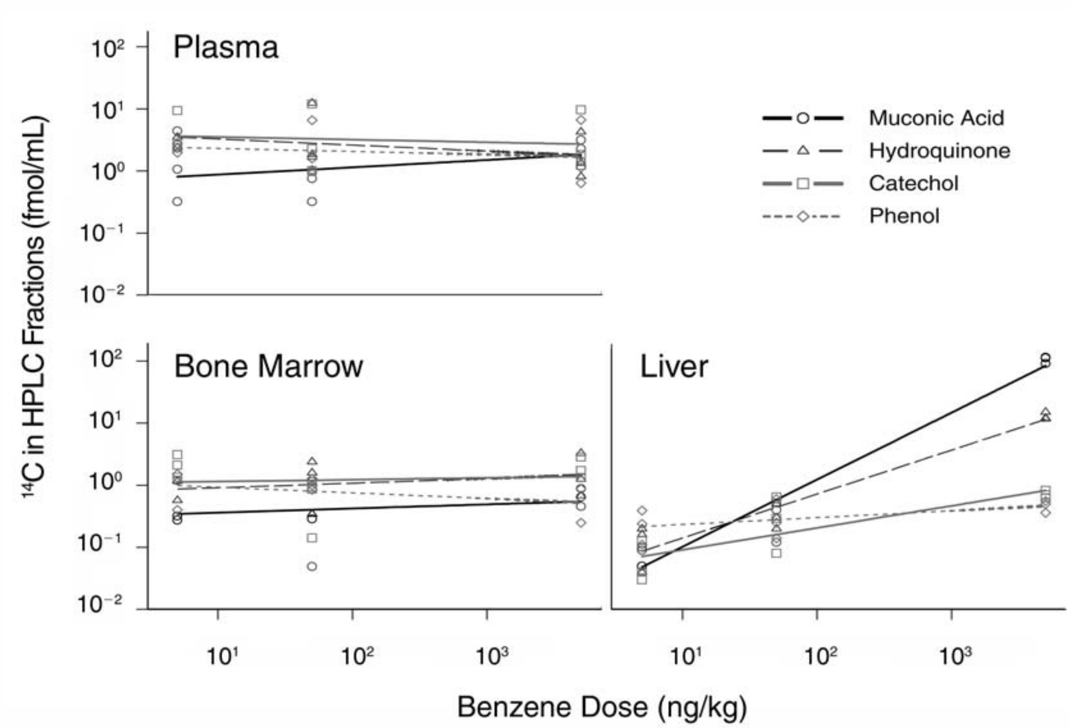

Figure 4. Dose response for four metabolites (catechol, hydroquinone, muconic acid, and phenol) in plasma, liver and bone marrow from B6C3F$_1$ mice. Each metabolite was quantitated by analyzing the radiocarbon content in HPLC fractions corresponding to elution time for well-characterized standards. Separate linear fits of log response on log dose were performed for each combination of tissue and metabolite. Lines show the estimated mean metabolite at each log dose.

the liver, muconic acid, catechol, and hydroquinone formation were dose dependent, but phenol formation was not. Estimates of the linear regression slopes and their 95% confidence intervals are given in Table 1. In plasma and bone marrow, none of the metabolites were found to be dose dependent. This finding may have been due in part to the low levels of metabolite found and the difficulty in distinguishing between doses.

Toxicokinetics of Low-Dose Benzene Metabolism

The kinetics of metabolite formation and clearance in plasma, liver, and bone marrow at 0 to 48 hours after IP administration of ^{14}C-benzene at a dose of 5 µg/kg body weight to B6C3F$_1$ mice are shown in Figure 5. The data are geometric means over time for four different metabolites (hydroquinone, catechol, phenol, and muconic acid); the AUC values for each plot are given in Table 2.

The AUCs demonstrate that of those tested hydroquinone and muconic acid are the major metabolites found in liver and bone marrow. Hydroquinone was the primary metabolite found in liver and plasma; muconic acid was the most prevalent metabolite in bone marrow. Phenol and catechol were present in lower amounts in all tissues. As evidenced by the low R^2 values and large confidence intervals for the metabolite levels, the AUC data varied considerably. This variation was consistently found for all metabolite analyses carried out in this study. The reasons are unknown but likely due to animal to animal variation and artifacts related to collection of HPLC peaks, (such as variation in isotope levels eluting with time, isotope memory in the hardware, and methods used to identify and collect peaks). Other factors include error in pipetting, weighing tissues, dosing animals, and extracting samples. Further work is necessary to understand the sources of this variation. Because each sample was measured repeatedly and intrasample variation never exceeded 5%, the cause is probably not variation in measurement of isotope levels by AMS.

RELATION BETWEEN DOSE AND MACROMOLECULAR ADDUCT FORMATION IN B6C3F$_1$ MICE

Both DNA and protein adducts from B6C3F$_1$ mice were quantitated over a dose range of 5 ng/kg to 500 mg/kg body weight. Samples were collected both 1 and 12 hours after dose administration. Two time points were used because a

Table 1. Estimates of Linear Regression of Log Metabolite Response[a] on Log Benzene Dose[b] for Plasma, Bone Marrow, and Liver 1 Hour After Exposure of B6C3F$_1$ Mice

	Slope Estimates ± SE	95% Confidence Interval	P Value	R^2
Plasma				
Muconic acid	1.08 ± 0.11	−0.13, 0.37	0.30	0.15
Hydroquinone	0.71 ± 0.11	−0.36, 0.16	0.39	0.13
Catechol	−0.04 ± 0.11	−0.31, 0.23	0.72	0.02
Phenol	−0.05 ± 0.10	−0.28, 0.18	0.64	0.03
Bone Marrow				
Muconic acid	0.07 ± 0.12	−0.22, 0.35	0.60	0.04
Hydroquinone	0.07 ± 0.10	−0.16, 0.31	0.48	0.09
Catechol	0.03 ± 0.11	−0.24, 0.30	0.80	0.01
Phenol	−0.09 ± 0.09	−0.33, 0.14	0.36	0.17
Liver				
Muconic acid	1.08 ± 0.08	0.88, 1.28	0.00	0.96
Hydroquinone	0.71 ± 0.07	0.54, 0.87	0.00	0.94
Catechol	0.35 ± 0.09	0.14, 0.57	0.01	0.68
Phenol	0.11 ± 0.06	−0.04, 0.26	0.12	0.31

[a] Metabolite data are femtomoles ^{14}C per milliliter HPLC fraction; see Figure 4.

[b] Mice were administered IP doses between 5, 50, and 5000 ng benzene/kg body weight.

Table 2. AUC Values[a] for Kinetics of Metabolites[b] in Plasma, Liver, and Bone Marrow After IP Exposure to Benzene[c] of B6C3F$_1$ Mice

	AUC Estimate ± SE	95% Confidence Interval	R^2
Plasma			
Muconic Acid	0.56 ± 0.13	0.18, 0.93	0.40
Hydroquinone	1.82 ± 0.49	0.42, 3.23	0.30
Catechol	0.27 ± 0.12	−0.08, 0.62	0.28
Phenol	0.26 ± 0.08	0.02, 0.50	0.38
Liver			
Muconic acid	55.90 ± 15.18	12.44, 99.36	0.59
Hydroquinone	148.97 ± 22.98	83.16, 214.79	0.81
Catechol	2.14 ± 0.65	0.29, 4.00	0.31
Phenol	3.01 ± 0.58	1.34, 4.68	0.73
Bone Marrow			
Muconic acid	15.69 ± 2.17	9.47, 21.90	0.46
Hydroquinone	6.08 ± 1.08	2.99, 9.17	0.51
Catechol	1.16 ± 0.32	0.26, 2.06	0.28
Phenol	2.82 ± 0.39	1.71, 3.93	0.24

[a] AUC values are the trapezoidal rule approximation to the AUC.

[b] Metabolite data are femtomoles ^{14}C per milliliter HPLC fraction; see Figure 5.

[c] Administered dose was 5 µg benzene/kg body weight.

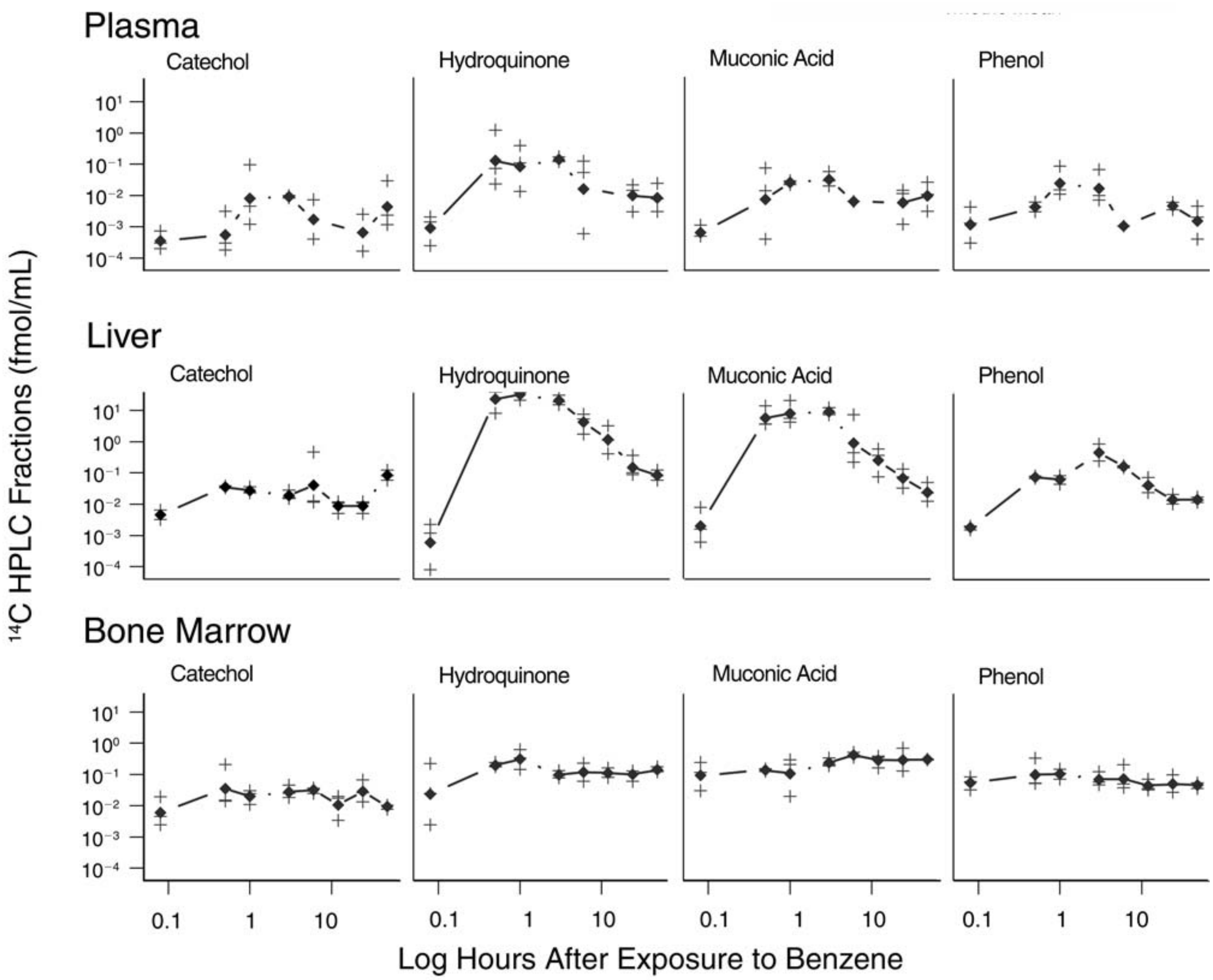

Figure 5. Kinetics of benzene metabolites in plasma, liver and bone marrow of B6C3F$_1$ mice. Log-transformed geometric means of metabolite concentrations are shown relative to log hours (0 to 48) after exposure to 5 µg benzene/kg body weight. Individual data points are also shown. Metabolites were quantified by measuring the amount of radiocarbon in HPLC fractions corresponding to the elution of well-characterized standards. Curves were generated by interpolating between the means. (+ Data Point, ◆ Geometric Mean)

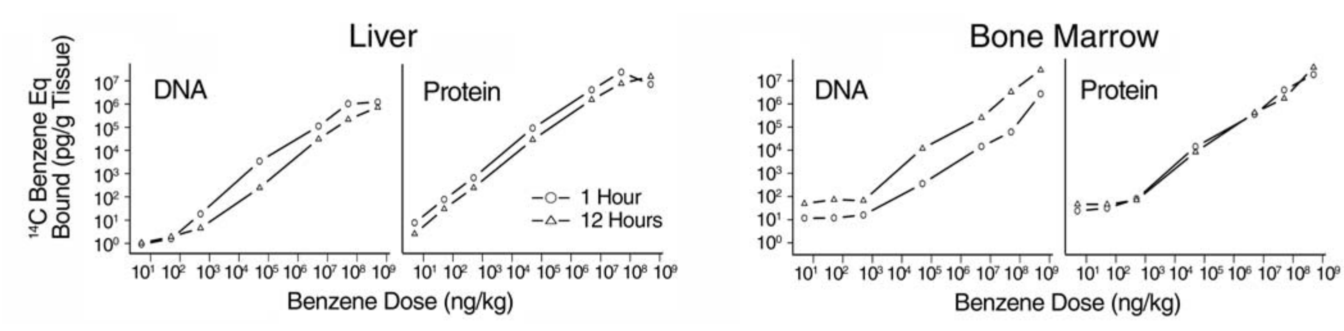

Figure 6. Benzene macromolecular adducts in liver and bone marrow of B6C3F$_1$ mice at 1 hour (○) and 12 hours (△) after exposure. Curves were generated by interpolating between the geometric means.

preliminary study by Creek and colleagues (1997) suggested that the kinetics of adduct formation between liver and bone marrow. In that study, adduct levels in liver reached maximal concentrations within a few hours of exposure while bone marrow adducts did not reach maximal concentrations until 6 to 12 hours after exposure.

Figure 6 shows the dose response of both DNA and protein adduct formation in liver and bone marrow over a dose range of 8 orders of magnitude, including doses relevant for human nonoccupational exposure to benzene. The data are log transformations of the adduct levels (picogram benzene equivalents per gram macromolecule) and doses (nanograms benzene per kilogram body weight) at 1 and 12 hours after exposure. Similar to the metabolite levels in urine, plasma, and tissues, protein and DNA adduct levels at both time points depended on dose level. Adduct levels for both protein and DNA in liver appeared to plateau at doses higher than 50 mg/kg body weight and tended to be higher 1 hour after exposure. In bone marrow, adduct levels were equivalent at both time points for protein and higher at 12 hours after exposure for DNA. This trend is clearly shown by comparing the confidence intervals for the difference between 1 hour and 12 hours after exposure (Figure 7).

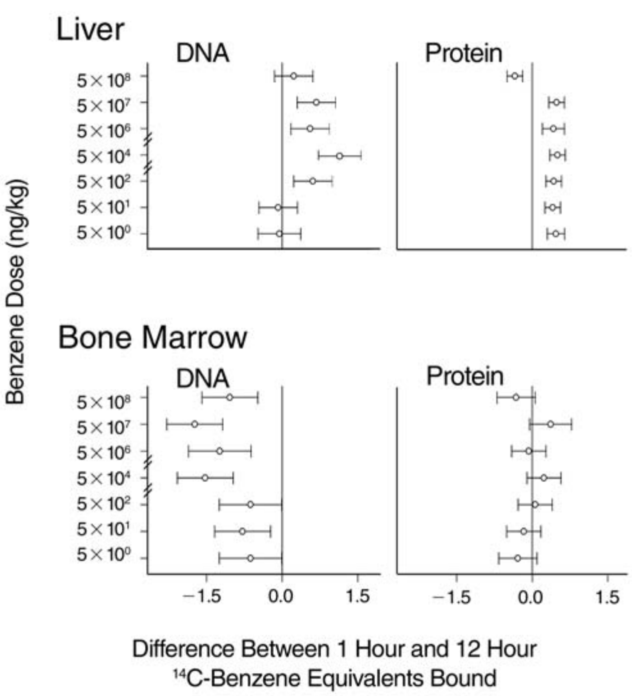

Figure 7. Differences between log ^{14}C-benzene equivalents bound at 1 hour and 12 hours as a function of dose in liver and bone marrow. Means are for DNA and protein adducts in liver and bone marrow. Circles represent the mean response at 1 hour minus the mean response at 12 hours for a given dose (milligrams benzene per kilogram body weight), tissue, and adduct. Lines represent individual 95% confidence intervals for the differences in means.

MACROMOLECULAR ADDUCT LEVELS AMONG RATS AND MICE BY AUC

B6C3F$_1$ mice, DBA/2 mice, C57BL/6 mice, and rats were administered 5 µg benzene/kg body weight intraperitoneally, and adduct levels were determined at various times afterward. Mean DNA and protein adduct data for bone marrow and liver are shown in Tables 3 and 4. Figure 8 shows the kinetics of DNA adducts in B6C3F$_1$ mice and rats. Consistent with previous work (Creek et al 1997), DNA and protein adducts in liver from all the mouse strains were detectable immediately after benzene administration and reached peak levels within 0.5 to 1 hour. In contrast, liver DNA and protein adduct levels in the rat continued to increase until 6 to 12 hours after exposure while bone marrow adduct kinetics were similar for the

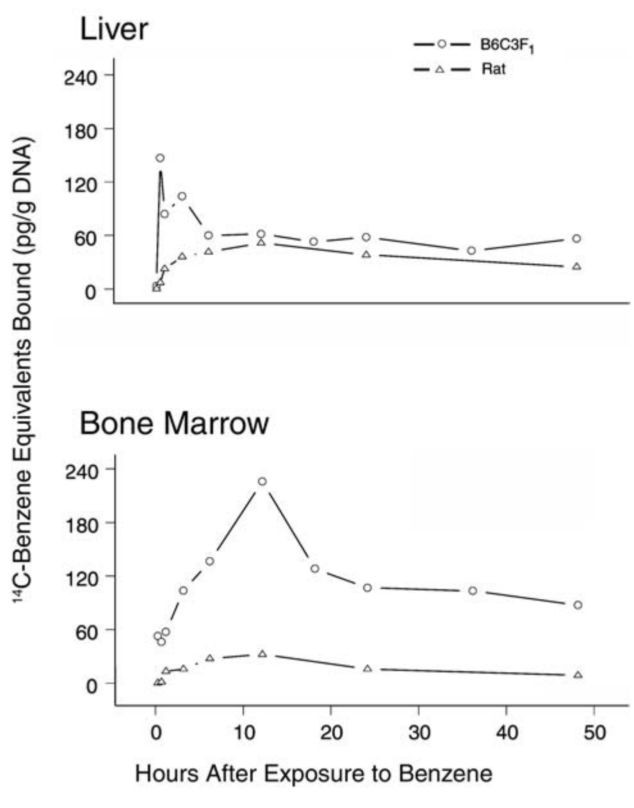

Figure 8. DNA adduct kinetics in B6C3F$_1$ mice and rats after IP exposure to benzene (5 µg/kg body weight). Geometric means of ^{14}C-benzene equivalents bound to liver and bone marrow DNA (picograms benzene per gram DNA) are shown over time in B6C3F$_1$ mice and rats are shown. Curves were generated by interpolating between the geometric means. Weighted averages for each time point were used to calculate the AUCs. (The curves are different from the curves used in the paper by Mani and colleagues [1998].)

Table 3. Means[a] and Estimated AUCs for Adducts[b] in Bone Marrow After IP Administration of Benzene[c]

	Protein				DNA			
	$B6C3F_1$	DBA/2	C57BL/6	Rat	$B6C3F_1$	DBA/2	C57BL/6	Rat
Time (hours)[d]								
< 0.08	422	54	16	17	53	19	30	1
0.5	923	348	282	10	46	−11	76	1
1	829	374	268	34	68	34	22	15
3	822	375	298	221	109	125	109	16
6	834	499	333	242	137	43	74	28
12	1,122	644	338	291	233	150	76	34
18					129			
24	824	342	279	166	111	124	108	17
36					108			
48	688	293	157	159	88	56	68	9
AUC Data								
AUC[e]	40,560	19,291	12,659	9,217	6,008	4,793	4,122	891
AUC − background[f]	21,910	17,783	12,170	9,121	4,655	4,793	4,122	891
SE[g]	1,656	1,985	617	981	427	1,050	1,005	86
R^2 [h]	0.91	0.64	0.89	0.82	0.75	0.52	0.29	0.82

[a] Most means are of three samples, some are of two samples. $B6C3F_1$ at 24 hours is represented by a single sample.

[b] Picograms ^{14}C-benzene adduct per gram DNA or protein.

[c] 5 µg benzene/kg body weight.

[d] Data for <0.08 hours represent a sample taken within five minutes after IP administration of benzene. The other sampling times represent nominal sampling times. Actual sampling times varied up to five minutes.

[e] AUC values are the trapezoidal rule approximation to the AUC.

[f] Values are the trapezoidal rule approximation to the AUC above the minimum value observed for that curve. Data <0 were replaced by 0.

[g] SE represents the standard error in estimating the trapezoidal rule approximation to the AUC.

[h] R^2 represents the proportion of the variance among rodents eliminated by fitting the AUC data to the model.

rats and mice. In all cases, levels of protein adducts in both liver and bone marrow were 10-fold to 20-fold higher than levels of DNA adducts.

Adduct levels measured at 0 to 48 hours after dosing were used to calculate AUCs. These DNA and protein adduct AUCs are estimates of the total reactive dose to each tissue and were used to compare the adduct levels between strains of mice and between mice and rats. The model used had an R^2 between 0.3 and 0.9, depending on the sample group. The data for 0.08 hours actually represents samplings immediately after dose administration and are thus an estimate of the lag time between dose administration and death. Although the lag varied among animals, it never exceeded five minutes.

Significant differences ($P < 0.05$) in estimated AUCs for bone marrow DNA adducts were detected among the animal species and strains sampled (Table 3). Without background subtraction, time-integrated DNA adduct levels in bone marrow were greatest in the $B6C3F_1$ mouse, followed by the DBA/2 mouse, C57BL/6 mouse, and rat (Table 3). With background subtraction, the AUC values for $B6C3F_1$ and DBA/2 mice were indistinguishable. Adjusted and nonadjusted AUCs for bone marrow DNA and protein adducts were on average seven and four times greater, respectively, in the $B6C3F_1$ mouse than in the rat. In general, mean AUCs of bone marrow protein adducts were approximately 3 to 10 times greater than mean DNA adduct AUCs, depending on the strain and species analyzed.

Liver macromolecular adducts presented a less clear picture than the bone marrow adducts (Table 4). Again, estimated AUCs for both protein and DNA adducts showed significant differences ($P < 0.05$) among the animal species and strains sampled, but the ordering differed depending on macromolecule assessed and whether an adjustment is made for the overall background observed in the experimental series. Mean AUCs of liver protein

Table 4. Means[a] and Estimated AUCs for Adducts[b] in Liver After IP Administration of Benzene[c]

	Protein				DNA			
	$B6C3F_1$	DBA/2	C57BL/6	Rat	$B6C3F_1$	DBA/2	C57BL/6	Rat
Time (Hours)[d]								
< 0.08	2	8	0	2	3	110	3	1
0.5	2,442	2,004	1,662	148	166	258	41	9
1	1,639	1,647	668	323	98	204	34	23
3	1,025	1,238	251	450	106	153	27	37
6	1,005	1,205	189	592	63	153	27	42
12	1,048	429	203	609	68	138	33	54
18	715				57			
24	1,043	410	202	479	59	128	28	39
36	396				43			
48	293	320	174	279	58	136	30	25
AUC Data								
AUC[e]	36,730	26,580	10,622	21,707	2,887	6,663	1,419	1,800
AUC – background[f]	36,669	26,289	10,622	21,689	2,772	1,514	1,419	1,799
SE[g]	5,372	3,018	854	2,383	265	138	106	140
R^{2}[h]	0.75	0.84	0.93	0.67	0.56	0.74	0.75	0.82

[a] Most means are of three samples, some are of two samples.

[b] Picograms ^{14}C-benzene adduct per gram DNA or protein.

[c] 5 µg benzene/kg body weight.

[d] Data for <0.08 hours represent a sample taken within five minutes after IP administration of benzene. The other sampling times represent nominal sampling times. Actual sampling times varied up to five minutes.

[e] AUC values are the trapezoidal rule approximation to the AUC.

[f] Values are the trapezoidal rule approximation to the AUC above the minimum value observed for that curve. Data <0 were replaced by 0.

[g] SE represents the standard error in estimating the trapezoidal rule approximation to the AUC.

[h] R^2 represents the proportion of the variance among rodents eliminated by fitting the AUC data to the model.

adducts, with and without background subtraction, were greatest for the $B6C3F_1$ mouse, followed by the DBA/2 mouse, rat, and C57BL/6 mouse (Table 4). This trend was nearly identical for bone marrow adduct AUCs (Table 3), except that the rat AUC was higher than the C57BL/6 mouse AUC. Without the background subtraction, AUCs of liver DNA adduct were greatest in the DBA/2 mouse followed by $B6C3F_1$ mouse, rat, and C57BL/6 mouse. AUCs of the $B6C3F_1$ mouse liver DNA adducts were approximately two-fold greater on average than that of the rat, whereas AUCs for the rat and C57BL/6 mouse were similar. With background subtracted, however, AUCs of liver DNA adducts were ordered identically among the mouse strains as for liver protein adducts and bone marrow protein and DNA adducts (ie, $B6C3F_1$ > DBA/2 > C57BL/6). In this case, however, the rat AUC was between B6C3F1 and DBA/2, rather than between DBA/2 and C57BL/6. The difference in ordering may have been due to scatter among individual data points given the dose used (5 µg benzene/kg body weight).

MACROMOLECULAR ADDUCTS IN RATS AND $B6C3F_1$ MICE AFTER LOW-DOSE BENZENE INHALATION

Administration of benzene has been carried out using a variety of exposure routes, principally IP and oral. In contrast, environmental exposure can occur not only orally, but also via inhalation. Thus, a preliminary study was designed to compare the kinetics of adduct formation and clearance in rodents using an IP and inhalation body burden. Additionally, this work initiated the development of methods of utilizing AMS to conduct low-dose inhalation studies.

In this study, we delivered radiolabeled benzene at an airborne concentration calculated to give a nominal internal dose equivalent to a single dose of 5 µg benzene per kg body weight. Rats were exposed nose-only to 0.1 ppm benzene for 20 minutes and $B6C3F_1$ mice to

0.1 ppm for 2.5 minutes using the chamber described in the Materials and Methods. Tables 5 and 6 show adduct levels measured at various times out to 24 hours for animals exposed to benzene by inhalation. For comparison, adduct levels after IP administration of benzene are given in Tables 3 and 4. After inhalation exposure, benzene formed both DNA and protein adducts in liver and bone marrow of both rodent species, which indicated that benzene was absorbed and metabolized. Levels of protein adducts were greater than levels of DNA adducts in both species, regardless of the tissue examined or the route of exposure (see Tables 3 through 6). Figures 9 and 10 show the data used to calculate the 24-hour AUC values for IP and inhalation exposures for both species. Adduct kinetics in $B6C3F_1$ mice were similar for both routes of exposure; generally, adduct levels peaked in liver at 1 hour after exposure and in bone marrow at 12 hours after exposure. In contrast, adduct kinetics in rats differed for the IP and inhalation exposures: in bone marrow, levels of both protein and DNA adducts increased until 6 to 12 hours after IP exposure, whereas adduct formation peaked within an hour of inhalation exposure or showed no clear peak at all.

Table 5. Means[a] and Estimated AUCs for Adducts[b] in Liver After Inhalation of Benzene[c]

	$B6C3F_1$ Mice		Rats	
	Protein	DNA	Protein	DNA
Time (hours)[d]				
0.08	375	8	68	66
0.16			305	31
0.5	1,988	90	123	57
1	1,647	77	35	17
3	1,344	69	51	17
6	1,455	65		22
12	803	30	40	19
24	863	21	35	13
AUC Data				
AUC[e]	25,349	1,002	1,081	481
SE[f]	2,466	148	142	173
R^2	0.75	0.60	0.79	0.21

[a] Means are of three samples.

[b] Picograms ^{14}C-benzene adduct per gram DNA or protein.

[c] 5 µg benzene/kg body weight.

[d] Data for 0.08 hours represent a sample taken within five minutes after benzene inhalation. The other sampling times represent nominal sampling times. Actual sampling times varied up to five minutes.

[e] AUC values are the trapezoidal rule approximation to the AUC.

[f] SE represents the standard error in estimating the trapezoidal rule approximation to the AUC.

Table 6. Means[a] and Estimated AUCs for Adducts[b] in Bone Marrow After Inhalation of Benzene[c]

	$B6C3F_1$ Mice		Rats	
	Protein	DNA	Protein	DNA
Time (hours)[d]				
0.08	5	6	55	−9
0.16			21	2
0.5	434		37	4
1			50	5
3	402	63	50	12
6	396	48		
12	669	200	37	−8
24	476	438	31	10
AUC Data				
AUC[e]	10,288	6,287	930	268
SE[f]	1,100	614	124	83
R^2	0.74	0.96	0.40	0.26

[a] Means are of three samples.

[b] Picograms ^{14}C-benzene adduct per gram DNA or protein.

[c] 5 µg benzene/kg body weight.

[d] Data for 0.08 hours represent a sample taken within five minutes after benzene inhalation. The other sampling times represent nominal sampling times. Actual sampling times varied up to five minutes.

[e] AUC values are the trapezoidal rule approximation to the AUC.

[f] SE represents the standard error in estimating the trapezoidal rule approximation to the AUC.

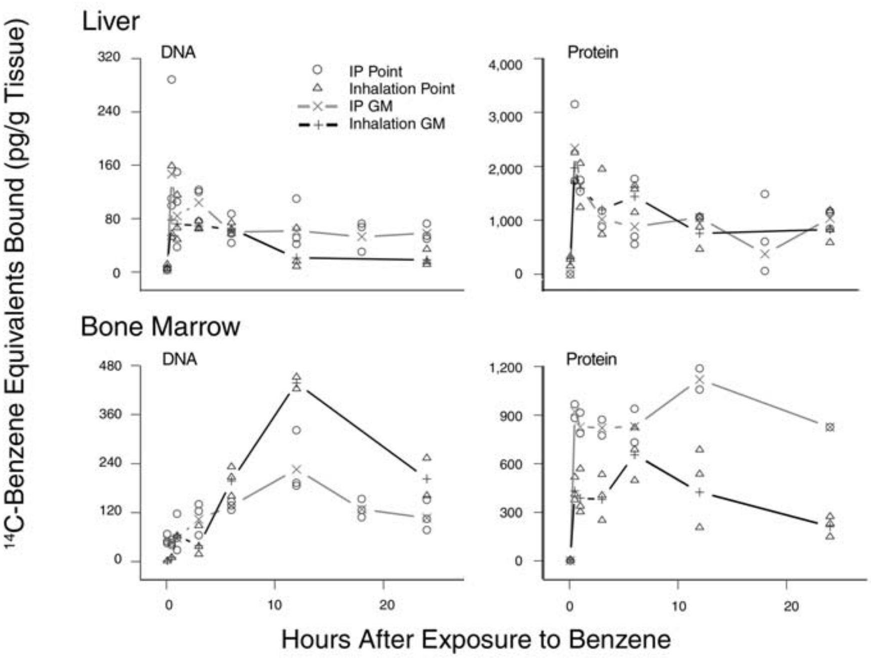

Figure 9. Change in benzene equivalents bound over time in B6C3F$_1$ mice after IP and inhalation exposures to benzene (at doses of 5 µg/kg body weight). Curves were generated by interpolating between the geometric means (GMs). Weighted averages for each time point were used to calculate the AUCs.

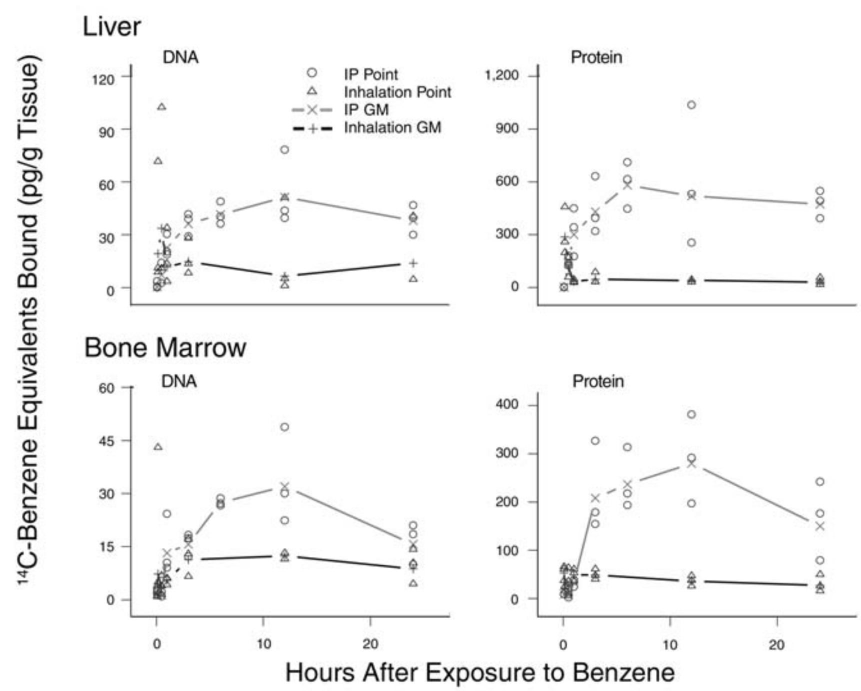

Figure 10. Change in benzene equivalents bound over time in the rat after IP and inhalation exposures to benzene (at doses of 5 µg/kg body weight). Curves were generated by interpolating between the geometric means (GMs). Weighted averages for each time point were used to calculate the AUCs.

Figure 11 shows the estimated differences between inhalation AUCs and IP AUCs and the 95% confidence intervals for those differences. Quantitatively, adduct levels were generally lower after inhalation than after IP administration in the rat and mouse. The B6C3F$_1$ mice yielded consistently greater adduct levels in both liver and bone marrow than the rat after inhalation of benzene (see Tables 5 and 6).

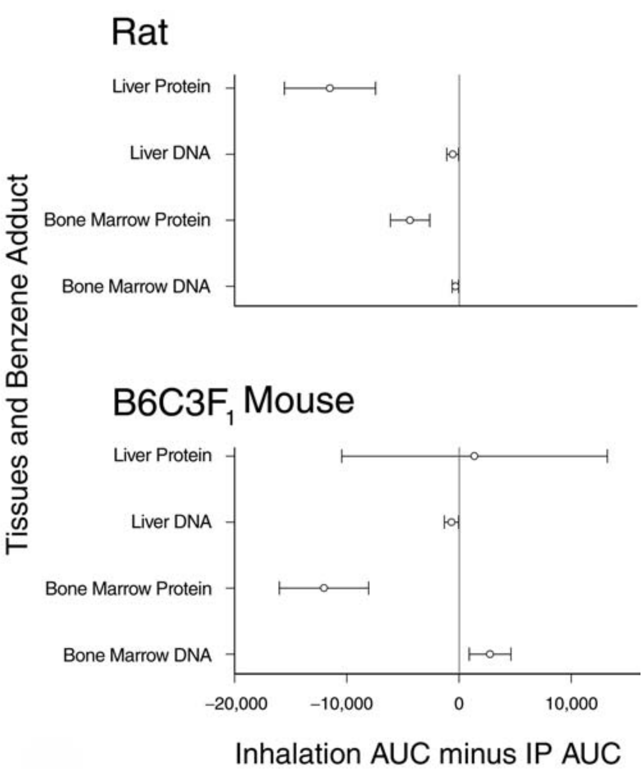

Figure 11. Estimated differences between responses to inhalation and IP exposures to benzene for rats and B6C3F$_1$ mice. AUC estimates of mean response from 0 to 24 hours after IP exposure were subtracted from estimates from 0 to 24 hours after inhalation exposure (differences represented by circles). Lines represent joint 95% confidence intervals for the difference in mean responses. Critical value for the confidence intervals was calculated using Sidak's method. Units are picograms of ^{14}C-benzene equivalents bound times hours per gram tissue.

DISCUSSION

The goal of this work was to investigate how benzene metabolism and macromolecular adduction relate to benzene dose. The dose range of interest varied from high doses used in traditional bioassays to low doses to which humans are exposed via their environment. We concentrated on macromolecular adducts and the metabolites believed to yield these adducts because they both can cause cell damage. DNA adducts are believed to be molecular lesions that lead to mutation and cancer. Protein adducts may also be involved in some types of cell damage (Epe et al 1990). Both types of macromolecular adduct are indicators of the reactive dose of carcinogen to the tissues and are also endpoints that can be quantified shortly after exposure has occurred. Measurement of these adducts has been central for determining exposure in the fields of molecular epidemiology and risk assessment (Golding and Watson 1999; Rappaport and Yeowell-O'Connell 1999).

We focused on liver, bone marrow, and plasma because liver is believed to be a primary site of benzene metabolism and bone marrow is a primary target organ for leukemia and other blood disorders (Snyder and Kalf 1994; Snyder and Hedli 1996). Also, reactive metabolites of benzene probably form in situ in bone marrow (Rushmore et al 1984; Eastmond et al 1987). Plasma was studied as a means of determining the circulating dose of benzene.

In order to measure the low concentrations of metabolites and adducts expected after low-dose exposure, we utilized AMS. This technique can quantify radiolabeled chemicals with attomole sensitivity and has been the standard for radiocarbon dating since the early 1980s (Vogel et al 1995). Although its use in biomedical fields is much more recent, initial studies have indicated that ^{14}C-labeled biomolecules can be quantified to levels of a few attomoles with high precision (Turteltaub and Vogel 1995; Vogel et al 1995).

Studies using AMS rely exclusively on measurement of radiocarbon levels in the biological material of interest. In this case we used isolated genomic DNA, isolated protein, or extracts of plasma, liver tissue, bone marrow, or urine. Some radiocarbon in individual samples may have been due to metabolic incorporation or noncovalent binding of benzene or its metabolites to DNA or protein. The DNA and protein used in this study were extensively purified by anion exchange chromatography and solvent extraction, however, and no radiocarbon was lost from samples via dialysis. No protein contamination or RNA contamination has been seen using these methods where DNA digestion has been carried out (Frantz et al 1995; Creek et al 1997; Kautiainen et al 1997; Mauthe et al 1999). Thus, while we cannot rule out the possibility of metabolic incorporation or noncovalent binding, we believe it is unlikely to have contributed substantially to the kinetics or binding seen in this study.

METABOLISM OF BENZENE TO REACTIVE INTERMEDIATES IN B6C3F$_1$ MICE

Our results show that at all doses, including those doses in the range of human exposure, benzene was rapidly absorbed and metabolized to reactive intermediates that bound both DNA and protein. DNA and protein adducts and individual benzene metabolites were present in all samples studied (plasma, liver, and bone marrow). Measured levels of metabolites (except those in plasma and bone marrow), DNA adducts, and protein adducts were dose dependent between the doses of 50 ng/kg and 50 mg/kg body weight. Neither metabolite profiles nor relative amounts of each metabolite changed as a function of dose. Whereas adducts were dose dependent, evidence of saturation in protein and DNA binding (plateauing of adduct levels) was found in liver at the higher doses (greater than 50 mg/kg body weight). The nature of the trend toward a plateau in hepatic adduct levels at higher doses was not addressed in this work, but it may be due to a dose-dependent increase in benzene clearance via expired air (as reported by Sabourin and colleagues 1987 and Mathews and coworkers 1998). The apparent trend toward flattening of the dose-response curves at lower doses of benzene, which indicate a loss of dose dependency), could have a variety of causes: the log-log transformation used to plot the data, artifacts of approaching the limit of detection, changes in metabolism or repair, or any combination of these factors. We made no attempt to characterize the chemical nature of the adducts or to determine the reason for the trends. Our main finding, that DNA adducts and protein adducts are present in liver and bone marrow at all doses, shows that reactive forms of benzene were present in the tissues even at low doses.

In some previous studies, DNA adducts in liver or bone marrow were not detectable after single doses of benzene but were detectable after twice-daily administration of benzene for several days (Reddy et al 1989, 1994; Pathak et al 1995; Bodell et al 1996). However, several other laboratories have reported the binding of radiolabeled benzene to DNA in various rat tissues after single treatment, (Lutz and Schlatter 1977; Arfellini et al 1985; Mazzullo et al 1989); these results support the findings reported here.

DISTRIBUTION OF REACTIVE BENZENE METABOLITES AMONG LIVER, BONE MARROW, AND URINE IN B6C3F$_1$ MICE

The toxicity of benzene is generally considered to be mediated by metabolites formed in the liver, which then distribute to other tissues where additional metabolism may occur (Greenlee et al 1981; Eastmond et al 1987; Snyder and Hedli 1996). Our studies in B6C3F$_1$ mice found muconic acid (an indicator of muconaldehyde) and hydroquinone, metabolites thought to be involved in benzene toxicity, in liver tissue in high concentration relative to the other metabolites there. Muconic acid was used as a marker for muconaldehyde production, as in previous studies (Parke and Williams 1953; Goldstein et al 1982). Metabolite profiles of plasma, liver, and bone marrow were similar, with both hydroquinone and muconic acid predominating over catechol and phenol. These data show that, at a low dose, the reactive benzene metabolites muconaldehyde and hydroquinone are present in tissues other than liver.

Although the metabolite AUCs have large standard errors, they suggest some useful conclusions. The total amounts of muconic acid and hydroquinone were greater, and they increased and peaked more rapidly in liver than in bone marrow or plasma. These findings and the fact that both metabolites were present in plasma are consistent with hepatic production of benzene metabolites and their transport to extrahepatic tissues, including bone marrow. The fact that metabolite levels were lower in plasma than in bone marrow is also consistent with some accumulation of these metabolites at low doses of benzene, as Sabourin and colleagues have suggested (1989). These results are also consistent with circulating benzene or benzene metabolites yielding reactive metabolites directly in the bone marrow (Eastmond et al 1987). This process would lead to higher levels of adducts in bone marrow than in liver and more time before maximal adduct concentrations are reached in bone marrow.

In urine, one of the major ^{14}C-labeled products found was an unidentified compound. We speculate that this radiolabeled compound was a contaminant, a unique metabolite, or a decomposition product of a benzene metabolite. For example, the compound could represent a benzene metabolite, such as phenyl mercapturic acid, arising via conjugation with glutathione. This could occur via the cyclohexadienyl pathway. However, our attempts to characterize this metabolite using mass spectrometry and HPLC coelution with an authentic phenyl mercapturic acid standard failed. In previous studies, Mathews and coworkers (1998) reported that hydroquinone glucuronide and phenyl sulfate were the major metabolites of benzene in urine of B6C3F$_1$ mice after oral administration of 0.1 mg benzene per kg body weight. Similarly, Sabourin and colleagues (1989) observed that hydroquinone glucuronide was the major metabolite, followed by phenyl sulfate, in urine of mice administered 1 to10 mg benzene per kg body weight by gavage. However, inhalation of 5 ppm benzene showed phenyl sulfate rather than hydroquinone glucuronide as the major product in mouse urine (Sabourin et al 1989). Thus it appears that at lower doses a different, presently unknown, metabolite seems to predominate. Further work is needed to determine the nature of this metabolite and whether it plays a role in genotoxicity.

RELATION BETWEEN METABOLITE LEVELS AND MACROMOLECULAR ADDUCT FORMATION IN B6C3F$_1$ MICE

The relation between benzene metabolism and formation of benzene adducts is not clear. The dose responses for protein and DNA adduct levels were consistent with the dose response for metabolites in urine and both suggest dose-dependent differences in benzene metabolism. Hydroquinone and muconic acid were the major metabolites in liver and bone marrow and hydroquinone levels were much higher in liver than in bone marrow. DNA adduct AUCs were greater in bone marrow than in liver, and the difference in the AUCs for protein adduct levels between the two tissues was relatively small. Interestingly, the muconic acid AUCs in liver and bone marrow were approximately equivalent, paralleling the protein adduct AUCs. In addition, the kinetics of metabolite formation paralleled adduct formation: metabolites in liver increased and peaked more rapidly than bone marrow; adduct levels also rose faster and peaked earlier in liver. Levay and Bodell (1992) have shown that the benzene metabolites hydroquinone, catechol, and 1,2,4-benzenetriol can interact synergistically to produce increased levels of DNA adducts, but we were unable to detect 1,2,4-benzenetriol in the samples analyzed here.

Further, in vitro treatment of mouse bone marrow with hydroquinone in another study resulted in DNA adduct levels similar to those observed in bone marrow after in vivo treatment with benzene (Levay et al 1993). Several studies suggest that hydroquinone is the principal benzene metabolite to lead to DNA adduct formation in bone marrow (Irons 1985; Smith et al 1989; Irons et al 1992). The results reported here from B6C3F$_1$ mice, however, are inconsistent with hydroquinone being the sole reactive metabolite without additional metabolism occurring in extrahepatic tissues. These results are consistent with the hypothesis that both hydroquinone and muconaldehyde are important reactive benzene metabolites and that hydroquinone undergoes extrahepatic metabolism to form macromolecular adducts (Levay et al 1993; Bodell et al 1994).

RELATION BETWEEN MACROMOLECULAR ADDUCT LEVELS AND TOXICITY IN RODENTS

Comparison of AUCs for both protein and DNA adducts in B6C3F$_1$ mice showed that levels were higher in bone marrow than in liver, which is consistent with target-organ susceptibility: benzene causes leukemia but not liver cancer. Both protein and DNA adducts among different strains of mice also showed differences compared with the rat. Macromolecular adduct kinetics in the DBA/2 mouse, C57BL/6 mouse, and rat are qualitatively similar. Some differences in the rates of formation and rates of clearance are evident, but these differences probably result from scatter among individual data and may not be biological in origin. Quantitatively, both protein and DNA adduct levels in bone marrow, the primary target organ of benzene toxicity, were significantly different with adduct levels greatest in the B6C3F$_1$ mice and lowest in the rats. The C57BL/6 and DBA/2 mice were intermediate at most time points and also had lower AUCs for both DNA and protein adducts. The largest difference was again clearly between the B6C3F$_1$ mice and the rats.

The differences observed in macromolecular binding of benzene among different mouse strains and rats may have been partially or entirely due to differences in the concentration of activated benzene encountered by the protein and DNA. This difference likely involved differences in benzene toxicodynamics and toxicokinetics. Dose-dependent effects on metabolizing enzymes or transport proteins could result in toxicodynamic differences: differences in the proportion of reactive or detoxified metabolites or changes in the amount of benzene or its metabolites that are delivered to tissues. Additionally, because CYP2E1 (cytochrome P450 2E1) is required for benzene metabolism (Seaton et al 1994), CYP2E1 activity in liver may be a major factor in interspecies and interindividual benzene toxicity (Sabourin et al 1987).

Previous work has shown that B6C3F$_1$ mice have a greater capacity to metabolize benzene and are more sensitive to the tumorigenic effects of benzene than rats (Sabourin et al 1987, 1989; Huff et al 1988). Further, some data suggest that because benzene induces greater levels of micronuclei in B6C3F$_1$ mice, the strain can metabolize benzene to a greater extent relative to C57BL/6 or DBA/2 mice (Sabourin et al 1989; Smith et al 1990; Witz et al 1990). Longacre and associates (1981) showed that tissue levels of covalently bound metabolites were higher in mouse strains that were more sensitive to benzene toxicity than in less-sensitive strains.

These data imply that AUCs of protein and DNA adducts in bone marrow are consistent with the capacity to bioactivate benzene and with benzene's tumorigenicity among these rodents (Huff et al 1988, 1989). This implication supports our hypothesis that greater risk is associated with higher adduct levels, which result from an ability to produce higher levels of reactive benzene metabolites. Analysis of bone marrow AUCs supports the hypothesis that B6C3F$_1$ mice should be the most sensitive and rats the least sensitive to benzene, with the other two mouse strains tested here (C57BL/6 and DBA/2) being intermediate.

INHALATION VERSUS IP EXPOSURE TO BENZENE

Route of exposure to benzene can also lead to differences in metabolism and, in turn, differences in formation of macromolecular adducts. Benzene adduct formation resulting from low-dose inhalation versus IP administration differed for rats and mice. AUCs for DNA and protein adducts in both liver and bone marrow were generally two to three fold greater after IP administration rather than after inhalation. When benzene is administered to achieve a similar nominal body burden, AUCs of DNA and protein adducts were greater in the B6C3F$_1$ mice than the rat. The kinetics of adduct formation and clearance was similar among exposure routes in B6C3F$_1$ mice but was qualitatively different for the rat. Judged by the adduct levels found in the rat, very little benzene was taken up or metabolized when administered by inhalation.

Measurement of total urinary ^{14}C-benzene equivalents by Sabourin and colleagues (1987) indicated that mice can metabolize approximately four times more inhaled benzene than rats. Inhalation of benzene produces 10 times more hydroquinone conjugates in B6C3F$_1$ mice than in rats. In a study by Medinsky et al (1989), the patterns of benzene metabolites seen in rats and B6C3F$_1$ mice were very different for inhalation and oral administration. Rats primarily formed the detoxification metabolite phenyl sulfate, whereas mice formed hydroquinone glucuronide and muconic acid in addition to phenyl sulfate. Thus, even at environmentally relevant doses of inhaled benzene, mice appear to metabolize benzene to reactive metabolites that in turn lead to higher levels of macromolecular adducts in tissues. Adducts thus appear to reflect the capacity to metabolize benzene across species, strains, and exposure routes. Additionally, route of exposure does affect adduct levels and thus could affect risk estimates; depending on the animal type, inhalation was either equivalent to or less effective than IP administration. What these results mean in terms of human exposure to benzene is unclear given the multiple routes of exposure that occur (predominantly via inhalation or dermal contact), but we cannot assume that these rodent data can be directly extrapolated to humans.

SUMMARY

The data presented here show that mice and rats can metabolize benzene effectively at all doses including those relevant to human exposure. The data are consistent with our hypothesis that DNA adduct levels reflect the ability to metabolize benzene to toxic metabolites. Similarly, the data suggest that adducts and benzene metabolites reflect target organ specificity and toxicity. Putatively toxic metabolites are formed and accumulate in plasma, liver, and bone marrow. DNA and protein adducts are dose dependent and present even at exposure levels equivalent to those humans encounter in the environment. Adduct levels are lower in rats relative to mice, and this difference reflects metabolic capacity. Further, route of exposure also can affect adduct levels. Thus, exposure route, kinetics, and metabolism will likely affect resulting estimates of risk and the use of animal models to estimate it.

IMPLICATIONS OF FINDINGS

DNA and protein adducts are likely present in all tissues, even at doses of benzene to which humans are exposed in the environment. To our knowledge, this is the first study to demonstrate that toxic metabolites of benzene are generated in liver, plasma, and bone marrow from exposures environmentally relevant to humans. Adduct levels are dose dependent and indicate metabolic capacity and target-organ specificity. Thus, if they can be quantified, benzene adducts are probably suitable for use in risk assessment. Responses are not linear across doses, however, and the dose responses for adduct formation and metabolite levels most likely plateau above 50 mg benzene/kg body weight. More data are needed to better define the shape of the dose response in various tissues.

Our adduct data suggest that route of exposure and genetic differences can also play a large role in benzene toxicity through differences in metabolism and possibly other factors. This conclusion is consistent with other data that indicate variability in metabolism and toxicity among rodents. Data for humans are thus needed to better judge the effects of benzene because how well rodent models can predict human risk is unclear. This uncertainty is highlighted by the work of Seaton and coworkers (1994, 1995), whose data on benzene metabolism suggest that risk depends on individual genotype.

The ultimate metabolite binding to DNA appears to be neither hydroquinone or muconaldehyde alone. Rather, muconic acid measurements indicate that the combination of hydroquinone and muconaldehyde more closely correlates with macromolecular adduct formation among mouse tissues. Even at low doses of benzene, the pathways of benzene metabolism appear to be qualitatively similar in rats and mice although there are clearly quantitative differences. Henderson has suggested that the qualitative similarity is true for all species (Henderson 1996). The quantitative differences do indicate that selection of the correct animal model is important in order to judge the human risk for benzene. Again, data in humans are needed to understand how to interpret animal data for risk assessment.

In most species that have been studied, a greater portion of the benzene is converted to hydroquinone and muconaldehyde if the dose is low rather than high. This trend is consistent with our adduct formation data and also appears to be true for humans, where both hydroquinone and muconic acid have been detected in the urine of exposed workers (Rothman et al 1998). The amounts of these two metabolites were lower in more highly exposed workers, which is consistent with findings in previous animal studies (Sabourin et al 1988a,b, 1989).

Finally, this work indicates that the use of AMS can help elucidate the effects of low doses of environmental toxicants. AMS can help to quantify metabolites and macromolecular adducts and to study the mechanisms by which toxicants exert their effects at low doses. More research needs to be devoted to understanding how to use AMS in these capacities.

ACKNOWLEDGMENTS

All AMS measurements were taken at the Center for Accelerator Mass Spectrometry at the Lawrence Livermore National Laboratory. The authors thank Kurt Haack, Dr John Vogel, Esther Fultz, Dr David Nelson, and Dr Karen Dingley for advice and assistance in conducting experiments. Inhalation studies were performed by Dr Janet Benson (Lovelace Respiratory Research Institute, New Mexico). In addition to funding from the Health Effects Institute, this study was conducted under the auspices of the US Department of Energy under contract W-7405-ENG-48 awarded to the Lawrence Livermore National Laboratory.

REFERENCES

Aksoy M, Erdem S. 1978. Follow up study on the development of leukemia in 44 pancytopenic patients with chronic exposure to benzene. Blood 52:285–292.

American Conference of Government and Industrial Hygienists. 1999. Guide to Occupational Exposure Values. ACGIH, Cincinnati OH.

Arfellini G, Grilli S, Colacci A, Mazzullo M, Prodi G. 1985. In vivo and in vitro binding of benzene to nucleic acids and proteins of various rat and mouse organs. Cancer Lett 28:159–168.

Bailer AJ. 1988. Testing for the equality of area under the curves when using destructive sampling techniques. J Pharmacokinet Biopharm 16:303–309.

Bailer AJ, Piegorsch WW. 1990. Estimating integrals using quadrature methods with an application in pharmacokinetics. Biometrics 46:1201–1211.

Bauer H, Demitriadis EA, Snyder R. 1989. An in vivo study of benzene metabolite DNA adduct formation in liver of male New Zealand rabbits. Arch Toxicol 63:209–213.

Bechtold WE, Sabourin PJ, Henderson RF. 1988. A reverse isotope method for determining benzene and metabolites in tissues. J Anal Toxicol 12:176–179.

Bodell WJ, Levay G, Pongracz K, Pathak DN. 1994. DNA adducts formed by peroxidase activation of benzene metabolites. Polycyclic Aromatic Compounds 6:1–8.

Bodell WJ, Pathak DN, Levay G, Ye Q, Pongracz K. 1996. Investigation of the DNA adducts formed in $B6C3F_1$ mice treated with benzene: Implications for molecular dosimetry. Environ Health Perspect 104:1189–1193.

Carere A, Antoccia A, Crebelli R, Di Chiara D, Fuselli S, Iavarone I, Isacchi G, Lagorio S, Leopardi P, Marcon F. 1995. [Exposure to benzene and genotoxic effects among filling station attendants.] Epidemiol Prev 19:105–119.

Cocheo V, Sacco P, Boaretto C, De Saegert E, Ballesta PP, Skov H, Goelen S, Gonzalezll N, Caracena AB. 2000. Urban benzene and population exposure. Nature 404:141–142.

Creek MR, Mani C, Vogel JS, Turteltaub KW. 1997. Tissue distribution and macromolecular binding of extremely low doses of [^{14}C]-benzene in $B6C3F_1$ mice. Carcinogenesis 18:2421–2427.

Cronkite EP. 1987. Chemical leukemogenesis: Benzene as a model. Semin Hematol 1:2–11.

Cronkite EP, Bullis JE, Inoue T, Drew RT. 1984. Benzene inhalation produces leukemia in mice. Toxicol Appl Pharmacol 75:358–361.

Cronkite EP, Drew RT, Inoue T, Hirabayashi Y, Bullis JE.1989. Hematotoxicity and carcinogenicity of inhaled benzene. Environ Health Perspect 82:97–108.

Department of Health and Human Services (US). 1986. Toxicology and Carcinogenesis Studies of Benzene in F344/N Rats and Mice. National Institutes of Health/National Toxicology Prog Tech Rep Ser 289, CAS 71-43-2. National Toxicology Program, Washington DC.

Eastmond DA, Smith MT, Irons RD. 1987. An interaction of benzene metabolites reproduces the myeolotoxicity observed with benzene exposure. Toxicol Appl Pharmacol 1:85–95.

Epe B, Harttig U, Stopper H, Metzler M. 1990. Covalent binding of estrogen metabolites to microtubular protein as a possible mechanism of aneuploidy induction and neoplastic cell transformation. Environ Health Perspect 88:123–127.

Frantz CE, Bangerter C, Fultz E, Mayer KM, Vogel JS, Turteltaub KW. 1995. Dose-response studies of MeIQx in rat liver and liver DNA at low doses. Carcinogenesis 16:367–374.

Golding BT, Watson WP. 1999. Possible mechanisms of carcinogenesis after exposure to benzene. IARC Sci Publ 150:75–88.

Goldstein BD. 1977. Hematotoxicity in humans. J Toxicol Environ Health 2(Suppl):69–105.

Goldstein BD, Witz G, Javid J, Amoruso M, Rossman T, Wolder B. 1982. Muconaldehyde: A potential toxic intermediate of benzene metabolism. In: Biological Intermediates II. Part A (Snyder R, Parke DV, Kocsis J, Jallow D, Gibson GG, Witmer CM, eds), pp 331–339. Plenum Press, New York NY.

Greenlee WF, Sun JD, Bus JS. 1981. A proposed mechanism of benzene toxicity: Formation of reactive intermediates from polyphenol metabolites. Toxicol Appl Pharmacol 59:187–195.

Henderson RF. 1996. Species differences in the metabolism of benzene. Environ Health Perspect 104:1173–1175.

Huff JE, Eastin W, Roycroft J, Eustis SL, Haseman JK. 1988. Carcinogenesis studies of benzene, methyl benzene, and dimethyl benzenes. Ann N Y Acad Sci 534:427–440.

Huff JE, Haseman JK, DeMarini DM, Eustis S, Maronpot RR, Peters AC, Persing RL, Chrisp CE, Jacobs AC. 1989. Multiple site carcinogenicity of benzene in Fischer 344 rats and B6C3F$_1$ mice. Environ Health Perspect 82:125–163.

Irons RD. 1985. Quinones as toxic metabolites of benzene. J Toxicol Environ Health 16:673–678.

Irons RD, Stillman WS, Colagiovanni DB, Henry VA. 1992. Synergistic action of the benzene metabolite hydroquinone on myelopoietic stimulating activity of granulocyte/macrophage colony-stimulating factor in vitro. Proc Natl Acad Sci U S A 89:3691–3695.

Kautiainen A, Vogel JS, Turteltaub KW. 1997. Trichloroethylene covalently binds macromolecules dose dependently in mice. Chem Biol Interact 106:109–121.

Kivistö H, Pekari K, Peltonen K, Svinhufvud J, Veidebaum T, Sorsa M, Aitio A. 1997. Biological monitoring of exposure to benzene in the production of benzene and in a cokery. Sci Total Environ 199:49–63.

Kolachana P, Subrahmanyam VV, Meyer KB, Zhang LP, Smith MT. 1993. Benzene and its phenolic metabolites produce oxidative DNA damage in HL60 cells in vitro and in the bone marrow in vivo. Cancer Res 53:1023–1026.

Laskin DE, Heck DE, Punjabi CJ, Laskin JD. 1987. Role of nitric oxide in hamatosuppression and benzene-induced toxicity. Environ Health Perspect 104:1283–1287.

Levay G, Bodell WJ. 1992. Potentiation of DNA adduct formation in HL-60 cells by combination of benzene metabolites. Proc Natl Acad Sci U S A 89:7105–7109.

Levay G, Pathak DN, Bodell WJ. 1996. Detection of DNA adducts in the white blood cells of B6C3F$_1$ mice treated with benzene. Carcinogenesis 17:151–153.

Levay G, Ross D, Bodell WJ. 1993. Peroxidase activation of hydroquinone results in the formation of DNA adducts in HL-60 cells, mouse bone marrow macrophages and human bone marrow. Carcinogenesis 14:2329–2334.

Longacre SL, Kocsis JJ, Snyder R. 1981. Influence of strain differences in mice on the metabolism and toxicity of benzene. Toxicol Appl Pharmacol 60:398–409.

Lutz WK, Schlatter CH. 1977. Mechanism of carcinogenic action of benzene: Irreversible binding to rat liver DNA. Chem Biol Interact 49:241–245.

Maltoni C, Ciliberti A, Cotti G, Conti B, Belpoggi F. 1989. Benzene, an experimental multipotential carcinogen: Results of the long-term bioassays performed at the Bologna Institute of Oncology. Environ Health Perspect 82:109–124.

Maltoni C, Conti B, Cotti G. 1983. Benzene: A multipotential carcinogen. Results of long term bioassays performed at the Bologna Institute of Oncology. Am J Ind Med 4:589–630.

Mani C, Vogel J, Turteltaub KW. 1998. Strain differences in the covalent binding of benzene to macromolecules: B6C3F1 vs C57BL/6 mice. 89th Annual Meeting of American Association for Cancer Research. March 28–April 1, 1998. American Association for Cancer Research, New Orleans LA.

Mathews JM, Etheridge AS, Mathews HB. 1998. Dose dependent metabolism of benzene in hamsters, rats and mice. Toxicol Sci 44:14–21.

Mathsoft. 1999. S-PLUS 2000 User's Guide. Mathsoft Data Analysis Products Division, Seattle WA.

Mauthe RJ, Dingley KH, Leveson SH, Freeman SP, Turesky RJ, Garner CR, Turteltaub KW. 1999. Comparison of DNA-adduct and tissue-available dose levels of MeIQx in human and rodent colon following administration of a very low dose. Int J Cancer 80:539–545.

Mazzullo M, Bartoli S, Bonora B, Colacci A, Grilli S, Lattanzi G, Niero A, Turina MP, Parodi S. 1989. Benzene adducts with rat and nucleic acids and proteins: Dose-response relationship after treatment in vivo. Environ Health Perspect 82:259–266.

Medinsky MA, Sabourin PJ, Lucier G, Birnbaum LS, Henderson RF 1989. A physiological model for simulation of benzene metabolism by rats and mice. Toxicol Appl Pharmacol 99:193–206.

Nillson RI, Nordliner RG, Tagesson C, Walles S, Jarvholm BG. 1996. Genotoxic effects in workers exposed to low levels of benzene from gasoline. Am J Ind Med 30:317–324.

Occupational Safety and Health Administration (US). 1987. Final rule on occupational exposure to benzene. Fed Regist 54:34660–34762.

Parke DV, Williams RT. 1953. Studies in detoxification 49. The metabolism of benzene containing [$^{14}C_1$]-benzene. Biochem J 54:231–238.

Pathak DN, Levay G, Bodell WJ. 1995. DNA adduct formation in the bone marrow of B6C3F_1 mice treated with benzene. Carcinogenesis 16:1803–1808.

Pinheiro JC, Bates DM. 2000. Mixed Effects Models in S and S-PLUS. Springer-Verlag, New York NY.

Pongracz K, Bodell WJ. 1996. Synthesis of N2-(4-hydroxyphenyl)-2′-deoxyguanosine-3′-phosphate: Comparison by 32P-postlabeling with the DNA adducts formed in HL-60 cells treated with hydroquinone. Chem Res Toxicol 9:593–598.

Rappaport SM, Yeowell-O'Connell K. 1999. Protein adducts as dosimeters of human exposure to styrene, styrene-7,8-oxide and benzene. Toxicol Lett 108:117–126.

Reddy MV, Blackburn GR, Schreiner CA, Mehlman MA, Mackerer CR. 1989. ^{32}P-analysis of DNA adducts in tissues of benzene-treated rats. Environ Health Perspect 82:253–257.

Reddy MV, Schultz SC, Blackburn GR, Mackerer CR. 1994. Lack of DNA-adduct formation in mice treated with benzene. Mutat Res 325:149–155.

Rinsky RA, Hornung RW, Landrigan PJ. 1989. Re: "Benzene and leukemia: A review of the literature and a risk assessment." Am J Epidemiol 129:1084–1086.

Rothman N, Bechtold WE, Yin SN, Dosemeci M, Li GL, Wang YZ, Griffith WC, Smith MT, Hayes RB. 1998. Urinary excretion of phenol, catechol, hydroquinone and muconic acid by workers occupationally exposed to benzene. Occup Environ Med 55:705–711.

Rothman N, Smith MT, Hayes RB, Traver RD, Hoener B, Campleman S, Li GL, Dosemeci M, Linet M, Zhang L, Xi L, Wacholder S, Lu W, Meyer KB, Titenko-Holland N, Stewart JT, Yin S, Ross D. 1997. Benzene poisoning, a risk factor for hematological malignancy, is associated with the NQO1 609C-->T mutation and rapid fractional excretion of chlorzoxazone. Cancer Res 57:2839–2842.

Rushmore T, Kalf G, Snyder R. 1984. Covalent binding of benzene and its metabolites to DNA in rabbit bone marrow mitochondria in vitro. Chem Biol Interact 49:133–154.

Sabourin PJ, Bechtold WE, Birnbaum LS, Lucier G, Henderson RF. 1988a. Differences in the metabolism and disposition of inhaled [3H]benzene by F344/N rats and B6C3F_1 mice. Toxicol Appl Pharmacol 94:128–140.

Sabourin PJ, Bechtold WE, Griffith WC, Birnbaum LS, Lucier G, Henderson RF. 1989. Effect of exposure concentration, exposure rate and route of administration on metabolism of benzene by F344 rats and B6C3F_1 mice. Toxicol Appl Pharmacol 99:421–444.

Sabourin PJ, Bechtold WE, Henderson RF. 1988b. A high pressure liquid chromatographic method for the separation and quantitation of water-soluble radiolabeled benzene metabolites. Anal Biochem 170:316–327.

Sabourin PJ, Chen BT, Lucier G, Birnbaum LS, Fisher E, Henderson RF. 1987. Effect of dose on the absorption and excretion of [^{14}C]benzene administered orally or by inhalation in rats and mice. Toxicol Appl Pharmacol 87:325–336.

Sabourin PJ, Muggenburg BA, Couch RC, Lefler D, Lucier G, Birnbaum, LS, Henderson RF. 1992. Metabolism of [^{14}C]benzene by cynomolgus monkeys and chimpanzees. Toxicol Appl Pharmacol 114:277–284.

Seaton MJ, Schlosser PM, Bond JA, Medinsky MA. 1994. Benzene metabolism by human liver microsomes in relation to cytochrome P450 2E1 activity. Carcinogenesis 15:1799–1806.

Seaton MJ, Schlosser P, Medinsky MA. 1995. In vitro conjugation of benzene metabolites by human liver: Potential influence of interindividual variability on benzene toxicity. Carcinogenesis 16:1519–1527.

Smith ET, Yager JW, Steinmetz KL, Eastmond DA. 1989. Peroxidase dependence metabolism of benzene's phenolic metabolites and its potential role in benzene's toxicity and carcinogenecity. Environ Health Perspect 82:23–29.

Smith MT. 1996. Overview of benzene-induced aplastic anaemia. Eur J Haematol 57:107–110.

Smith MT, Robertson ML, Yager JW, Eastmond DA. 1990. Role of metabolism in benzene-induced myelotoxicity and leukemogenesis. Prog Clin Biol Res 340B:125–136.

Snyder R, Hedli CC. 1996. An overview of benzene metabolism. Environ Health Perspect 104:1165–1171.

Snyder R, Kalf A. 1994. A perspective on benzene leukemogenesis. Crit Rev Toxicol 24:177–209.

Snyder R, Kocsis JJ. 1975. Current concepts of chronic benzene toxicity. CRC Crit Rev Toxicol 3:265–288.

Snyder R, Lee EW, Kocsis JJ. 1978. Binding of labeled benzene metabolites to mouse liver and bone marrow. Res Commun Chem Pathol Pharmacol 20:191–194.

Snyder R, Witz G, Goldstein BD. 1993. The toxicology of benzene. Environ Health Perspect 100:293–306.

Sun JD, Medinsky MA, Birnbaum LS, Lucier G, Henderson RF. 1990. Benzene hemoglobin adducts in mice and rats: Characterization of formation and physiological modeling. Fundam Appl Toxicol 15:468–475.

Tompa A, Major J, Jakab MG. 1994. Monitoring of benzene-exposed workers for genotoxic effects of benzene: Improved-working-condition-related decrease in the frequencies of chromosomal aberrations in peripheral blood lymphocytes. Mutat Res 304:159–165.

Tuo JS, Deng XS, Loft S, Poulsen HE.1999. Dexamethasone ameliorates oxidative DNA damage induced by benzene and LPS in mouse bone marrow. Free Radic Res 30:29–36.

Turteltaub KW, Vogel JS. 1995. Application of accelerator mass spectrometry in toxicology: A highly sensitive tool for low-level isotope measurements. In: Mass Spectrometry in the Biological Sciences (Burlingame AL, Carr SA, eds), pp 477–495. Humana Press, Totawa NJ.

Turteltaub KW, Vogel JS, Frantz C, Felton JS, McManus M. 1995. Assessment of the DNA adduction and pharmacokinetics of PhIP and MeIOx in rodents at doses approximating human exposure using the technique of accelerator mass spectrometry (AMS) and 32P-postlabeling. Princess Takamatsu Symp 23:93–102.

Turteltaub KW, Vogel JS, Frantz CE, Fultz E. 1993. Studies on DNA adduction with heterocyclic amines by accelerator mass spectrometry: A new technique for tracing isotope-labeled DNA adduction. In: Postlabeling Methods for Detection of DNA Adducts (Phillips DH, Castegnaro M, Bartsch H, eds), pp 293–300. International Agency for Research on Cancer, Lyon, France.

Vogel JS. 1992. Rapid production of graphite without contamination for biological AMS. Radiocarbon 34:344–350.

Vogel JS, Turteltaub KW, Finkel R, Nelson DE. 1995. Accelerator mass spectrometry: Isotope identification at attomole sensitivity. Anal Chem 67:353A–359A.

Wallace LA. 1989. The exposure of the general population to benzene. In: Benzene: Occupational and Environmental Hazards. Scientific Update (Mehlman MA, ed), pp 113–130. Princeton Scientific Publishers, Princeton NJ.

Wallace L. 1996. Environmental exposure to benzene: An update. Environ Health Perspect 12:1129–1136

Wallace L, Pellizzari E, Hartwell T, Rosenzweig M, Erickson M, Sparacino C, Zelon H. 1984. Personal exposure to volatile organic compounds. Direct measurement in breath zone air, drinking water, food and exhaled breath. Environ Res 35:293–319.

Witz G, Kirley TA, Maniara WM, Mylavarapu VJ, Goldstein BD. 1991. The metabolism of benzene to muconic acid, a potential biologic marker of benzene exposure. Adv Exp Med Biol 283:613–618.

Witz G, Maniara W, Mylavarapu V, Goldstein BD 1990. Comparative metabolism of benzene and trans,trans-muconaldehyde to trans,trans-muconic acid in DBA/2N and C57BL/6 mice. Biochem Pharmacol 40:1275–1280.

Yeowell-O'Connell K, Rothman N, Smith MT, Hayes RB, Li G, Waidyanatha S, Dosemeci M, Zhang L, Yin S, Titenko-Holland N, Rappaport SM. 1998. Hemoglobin and albumin adducts of benzene oxide among workers exposed to high levels of benzene. Carcinogenesis 9:1565–1571.

APPENDIX AVAILABLE ON REQUEST

The following appendix is available by contacting the Health Effects Institute. Please give the author name, full title and number of the report.

Appendix A. Row Data for Studies.

ABOUT THE AUTHORS

Kenneth W Turteltaub received a PhD in toxicology from Iowa State University in 1987. He was a postdoctoral fellow with Dr James Felton from 1987 to 1990 in the Biomedical and Environmental Sciences Program at the Lawrence Livermore National Laboratory. Currently Dr Turteltaub is the head of the Molecular Toxicology Group Biology and Biotechnology Research Program of the Lawrence Livermore National Laboratory and holds an adjunct appointment in pharmaceutical chemistry at the University of California, San Francisco. His primary research interests involve the mechanisms of carcinogenesis and the development of biomedical applications of accelerator mass spectrometry.

Chitra Mani received a bachelor's degree from Madras University, India. She received a PhD in biomedical sciences from Worcester Polytechnic Institute, Massachusetts, in 1993. Her thesis focused on the bioactivation of the binding of tamoxifen to microsomal proteins in liver. She continued on to a postdoctoral fellowship at Stanford University and joined Dr Turteltaub's group at Lawrence Livermore National Laboratory in 1995. She is presently a scientist at Pharmacyclics, a pharmaceutical company in Sunnyvale, California.

OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

Mani C, Freeman S, Nelson D, Vogel SJ, Turteltaub KW. 1999. Species and strain differences in the macromolecular binding of extremely low doses of [14C]benzene in rodents. Toxicol Appl Pharmacol 159:83–90.

Creek MR, Mani C, Vogel JS, Turteltaub KW. 1998. Tissue distribution and macromolecular binding of extremely low doses of [^{14}C]-benzene in $B_6C_3F_1$ mice. Carcinogenesis 18:2421–2427.

Turteltaub KW, Mani C, Dingley KH, Bench G, Mauthe RJ. 1998. DNA adducts using accelerator mass spectrometry. In: Biomarkers (Mendelsohn ML, Mohr LC, Peeters JP, eds), pp 99–114. Joseph Henry Press, Washington DC.

ABBREVIATIONS AND OTHER TERMS

AMS	accelerator mass spectrometry
attomole	10^{-18} mole
AUC	area under the curve
CO_2	carbon dioxide
DTT	dithiothreitol
EC	enzyme classification number
EPA	Environmental Protection Agency (US)
femtomole	10^{-15} mole
GLS	generalized least squares
HPLC	high-performance liquid chromatography
IP	intraperitoneal
MOPS	3-(*N*-morpholino)propanesulfonic acid
PCA	perchloric acid
RNase	ribonuclease
TBAHS	tetrabutylammonium hydrogen sulfate

COMMENTARY
Health Review Committee

INTRODUCTION

In humans and other species, chronic exposure to high concentrations of benzene induces a spectrum of conditions, including cancer. The effects of human exposure to benzene at the low levels found in ambient air are not well understood, however. One of the major reasons for this lack of understanding is the dearth of sensitive and accurate assays of benzene metabolites for these low concentrations. Consequently, the shape of the dose-response curve describing health effects at low benzene concentrations—an issue central to assessment of human risk from benzene—remains unresolved.

HEI initiated a research program in the 1990s to address the need for knowledge about health effects resulting from exposure to ambient levels of toxic air pollutants, including benzene. Request for Applications (RFA*) 93-1, *Novel Approaches to Extrapolation of Health Effects for Mobile Source Toxic Air Pollutants*, was part of that program. In response to this RFA, Dr Kenneth Turteltaub and his colleague Dr Chitra Mani proposed to investigate benzene metabolism in rodents over an eight log-dose range, encompassing concentrations close to those to which humans are exposed in ambient air (typically 1 to 10 ppb, equivalent to 3.2 to 32 $\mu g/m^3$ [Wallace 1996]). To achieve the sensitivity required to investigate the effects of low-level exposure to benzene, the investigators proposed to use accelerator mass spectrometry (AMS) coupled with high-performance liquid chromatography (HPLC). The HEI Health Research Committee recommended funding the proposal because it thought the study could yield important information about the metabolism of benzene at low-exposure doses. Because benzene itself is not thought to be the toxic agent, the Committee reasoned that obtaining quantitative data about the metabolism of benzene at different exposure levels would provide information relevant to understanding the health effects of benzene at these different levels of exposure.†

*A list of abbreviations and other terms appears at the end of the Investigators' Report.

† Dr Turteltaub's 3-year study, *Benzene Metabolism at Doses Relevant to Human Urban Air Exposure*, began in April 1995. Total expenditures were $717,150. The draft Investigators' Report from Turteltaub and Mani was received for review in November 2000. A revised report, received in August 2001, was accepted for publication in September 2001. During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and in the Review Committee's Commentary.

Dr Turteltaub's draft Investigators' Report underwent external peer review under direction of the HEI Health Review Committee, the members of which discussed the report and the reviewers' critiques and prepared the Commentary. This Commentary is intended to aid HEI sponsors and the public by highlighting the strengths of the study, pointing out alternative interpretations, and placing the report into scientific perspective. During the review of Dr Turteltaub's study, the HEI Review Committee and the investigators exchanged comments and clarified issues in the Investigators' Report and in the Commentary.

SCIENTIFIC BACKGROUND

BENZENE METABOLISM AND MECHANISM OF TOXIC EFFECTS

Exposure to benzene can be toxic to bone marrow and to cells derived from bone marrow in humans and other species. Depending on the level and duration of exposure, a spectrum of conditions including pancytopenia, aplastic anemia, and acute myeloid leukemia can be induced in humans (reviewed in Goldstein and Witz 2000). In other species, controlled exposure to high levels of benzene results in tumor formation at many sites but does not induce leukemias (International Agency for Research on Cancer 1982; Huff et al 1989).

Studies suggest that benzene must be metabolized to induce toxic effects (reviewed in Snyder and Hedli 1996). Some benzene metabolism does take place in the target organ (the bone marrow) but most occurs in the liver via multiple pathways. The major mechanism of benzene metabolism in the liver is oxidation by the cytochrome P450 2E1 monooxygenase system. Figure 1 in the Investigators' Report depicts the principal pathways of benzene metabolism and the metabolites formed. One of the key early intermediates in benzene metabolism is benzene oxide, a highly reactive epoxide, which spontaneously rearranges to form phenol. Phenol in turn can be oxidized by cytochrome P450 enzymes to catechol (1,2-dihydroxybenzene), hydroquinone (HQ, or 2,4-dihydroxybenzene), or 1,2,4-trihydroxybenzene (also referred to as benzene triol, or BT). Benzene oxide can also be metabolized by opening the benzene ring, producing metabolites that include *trans,trans*-muconic acid (*t,t*-MA). Benzene oxide also reacts with glutathione to form *S*-phenylmercapturic acid (*S*-PMA). In bone marrow, myeloperoxidase catalyzes the further oxidation of ring-hydroxylated benzene metabolites to highly reactive quinones such as 1,2- and 1,4-benzoquinone (1,2- and 1,4-BQ). The highly reactive electrophilic metabolites of benzene, such as benzene oxide and the quinones, can form covalent adducts with nucleophilic sites on protein and DNA macromolecules.

The mechanism of benzene toxicity remains elusive despite extensive research. Several mechanisms have been proposed. One widely held hypothesis is that certain benzene metabolites (including benzoquinone, hydroquinone, and products of benzene triol) produce reactive oxygen species that interact with cellular macromolecules (Smith 1996). This interaction may result in genotoxic events. Another interaction that has been described is inhibition (by activated benzene metabolites) of the activity of the nuclear enzyme topoisomerase II (Chen and Eastmond 1995). Because this enzyme is involved in stabilizing the chromosomal structure, inhibiting its activity may induce chromosomal abnormalities (Chen and Eastmond 1995; Eastmond et al 2001). A combination of ring-hydroxylated compounds may also be the toxic agent of benzene (Eastmond et al 1987). Another hypothesis is that muconaldehyde (the precursor of *t,t*-MA), possibly in combination with other metabolites (Witz et al 1996), plays a role in benzene's toxicity by inducing oncogene expression (Ho and Witz, 1997).

ASSESSMENT OF RISK

Studies of occupational epidemiology have established that benzene can induce leukemia, and the US Environmental Protection Agency (EPA) has classified benzene as a known human carcinogen (EPA 1998a). The US Occupational Safety and Health Administration regulates benzene levels in the workplace, and has set a workplace standard of 1.0 ppm as an 8-hour time weighted average (OSHA 1987). In addition, the American Conference of Government and Industrial Hygienists has recommended a threshold limit value for occupational exposure of 0.5 ppm as an 8-hour time weighted average (ACGIH 1999) and the US National Institute of Occupational Safety and Health has recommended a workplace benzene standard of 0.2 pm (Goldstein and Witz 2000).

Little direct information is available about the human health risk of exposure to typical environmental levels of benzene. Thus, risk estimates have been based on extrapolating data obtained in other types of studies, low-dose exposures of nonhuman species and high-dose occupational exposures of humans. How accurately such studies model exposure of humans to low doses, however, is unclear. First, extrapolation to humans from controlled low-dose exposure studies of other species (especially rodents) is difficult because species, and different strains within a species, differ in their responses to benzene (Longacre et al 1981; Witz et al 1990; Huff 1993). For example, $B6C3F_1$ mice are more sensitive than Fischer rats to the hematotoxic and carcinogenic effects of chronic exposure to benzene (Huff 1993). In addition, studies prior to this one have reported that levels of specific metabolites differ among species and in different strains within a species (Longacre et al 1981; Sabourin et al 1988a; Witz et al 1990; Henderson 1996). These quantitative differences may be critical, and the differences in cancer susceptibility among different animals may be due to the relative amounts of different metabolites formed.

Second, extrapolating results of high-dose exposure of humans to benzene in order to predict effects of low-dose exposure is also difficult. In the absence of data on low-dose exposures, such extrapolation assumes linear relations among exposure level, metabolite mixture, and health outcome. High-dose and low-dose exposures of humans to benzene may result in different mixtures of metabolites, however, as has been described for rats and mice and in an epidemiologic study: high levels of benzene generate relatively less hydroquinone than lower levels (Henderson 1996; Rothman et al 1998). These findings suggest that extrapolation from high to low dose should consider metabolic patterns at various exposure levels. These metabolite patterns should be more closely related than benzene exposure levels to health outcomes.

1998, the EPA published *Carcinogenic Effects of Benzene: An Update*, its most recent exhaustive review of pertinent studies of benzene's cancer effects (EPA 1998a). Regarding an estimate of risk from environmental exposure to benzene, the document states that "there is not sufficient evidence…to reject a linear dose-response curve for benzene in the low-dose region, nor is there sufficient evidence to demonstrate that benzene is, in fact, nonlinear in its effects." Thus, "the EPA default approach of using a model with low-dose linearity is still recommended. Of the various approaches employing a linear assumption, the [lifetime] risk at 1 ppm [of benzene in air] ranges from 7.1 $\times 10^{-3}$ to 2.5×10^{-2}, within which any calculated unit risk estimate would have equal scientific validity." The EPA has produced a draft of a similarly comprehensive document on the noncancer effects of benzene (EPA 1998b).

Although the linear model is currently used in estimating risk, arguments exist for a model assuming either a supralinear or sublinear dose response. Improving the accuracy of benzene risk assessment at environmental exposure levels requires more information of several types: information about the mechanism of induction of benzene-induced leukemia and other benzene-induced disorders; information needed to characterize the specific mixture of benzene metabolites delivered from liver to bone marrow, the fate of these metabolites in the bone marrow, and ultimately, the role of specific metabolites in development of blood abnormalities; and information about biomarkers of exposure as well as effect.

TECHNICAL EVALUATION

AIMS

1. *To determine how dose influences benzene metabolism in mice.* The investigators administered 5 ng to 500 mg ^{14}C-benzene per kg body weight to B6C3F$_1$ mice via intraperitoneal (IP) injection. They used HPLC to separate and identify several benzene metabolites (focusing on hydroquinone, MA, catechol, and phenol) in urine, plasma, liver, and bone marrow up to 48 hours after injection. They then used AMS to measure levels of ^{14}C-metabolites.
2. *To compare the formation of DNA and protein adducts with the profile of other metabolites over a wide range of benzene doses.* The investigators initially assessed the kinetics of forming DNA and protein adducts in B6C3F$_1$ mice after IP injection of a single dose of ^{14}C-benzene (5 µg per kg body weight). Using AMS, they measured adduct formation in liver and bone marrow up to 48 hours after injecting benzene. They also measured adduct formation over time at a range of ^{14}C-benzene doses (5 ng to 500 mg per kg body weight).
3. *To compare benzene adduct formation among different rodents after dosing with low doses of benzene.* Using AMS, the investigators measured formation of DNA and protein adducts in liver and bone marrow up to 48 hours after IP injection of 5 µg/kg benzene to mice (B6C3F$_1$, DBA/2, C57BL/6) and to Fischer rats.
4. *To develop methods for measuring DNA and protein adducts after inhalation of low doses of benzene.* The investigators conducted a pilot study to develop a method for exposing mice and Fischer rats by inhalation to an approximate body burden of 5 µg benzene per kg body weight. They then compared formation of DNA and protein adducts after inhalation and after IP injection of the same dose of benzene.

METHODS

Exposure to Benzene

The investigators used male B6C3F$_1$, DBA/2, and C57BL/6 mice (30 g) and male Fischer rats (200 g). In most experiments, they exposed the animals to various concentrations of benzene via IP injection: each animal received 200 µL corn oil containing 0.1 µCi of ^{14}C-benzene diluted with nonradioactive benzene. According to the investigators, the radioactive benzene was 99% radiopure and contained no contaminating peaks when assayed by HPLC. Control animals were given corn oil only. For toxicokinetic studies, the investigators gave animals IP injections of 5 µg ^{14}C-benzene per kg body weight in corn oil (200 µL/animal) and killed the animals at intervals from 0 to 48 hours after injection.

In some experiments, the investigators exposed rats and mice to 5 µg benzene per kg body weight by nose-only inhalation. The duration of exposure was based on each species' weight (rat, 200 g; mouse, 30 g) and volume of air delivered per minute (0.2 L for rats, 0.04 L for mice). Rats were exposed to 0.1 ppm ^{14}C-benzene (0.09 µCi/animal) for 20 minutes via nose-only inhalation in a chamber at the Lovelace Respiratory Research Institute (Albuquerque NM) under the supervision of Dr Janet Benson. Mice were exposed to 0.1 ppm benzene for 2.5 minutes via nose-only inhalation in a chamber constructed in the investigators' laboratory. The concentration of benzene to which the rodents were exposed was continuously monitored by online infrared spectroscopy.

Separation and Analysis of Benzene Metabolites

Turteltaub and Mani separated and quantified individual benzene metabolites in urine and in ethyl-acetate extracts of plasma, liver, and bone marrow by HPLC as described by Sabourin and colleagues (1988a). Metabolite standards were obtained either from Sigma Chemical Company (catechol, hydroquinone, MA, and phenol; > 99% pure) or were the gift of Dr William Bechtold, Lovelace Respiratory Research Institute (hydroquinone sulfate, phenyl glucuronide, and phenyl sulfate). Urinary metabolites were separated and quantified by HPLC as described in Sabourin and colleagues (1988b). Data were normalized to the recoveries of an internal standard and were expressed as femtomole ^{14}C-labeled metabolite per mL HPLC eluant (Bechtold et al 1988).

Measurement of Adducts

The investigators determined the amount of benzene-derived DNA and protein adducts, which they extracted and purified from liver and bone marrow of B6C3F$_1$ mice. DNA and protein were extracted at 1 and 12 hours after IP injection of 5 ng to 500 mg benzene per kg body weight. The investigators used two time points because they had previously found differences in the kinetics of adduct formation between liver and bone marrow: adduct burden in liver reached maximum levels within a few hours of exposure, whereas adduct burden in bone marrow reached maximum levels 6 to 12 hours after exposure (Creek et al

1997). To compare adduct formation among rats and mice, the investigators determined adduct levels in liver and bone marrow at various times up to 48 hours after IP injection of 5 µg benzene per kg body weight into B6C3F$_1$, DBA/2, and C57BL/6 mice and Fischer rats.

AMS Analysis

The investigators assessed the radiocarbon content of samples by AMS. To samples containing small amounts of carbon, such as extracted DNA adducts and HPLC fractions from liver, plasma, and bone marrow, they added the carbon-containing compound tributyrin as a carrier to improve the efficiency of sample handling. Samples were dried in a vacuum centrifuge and were then converted to graphite using a two-step process (Vogel 1992). Briefly, the dried samples were oxidized to carbon dioxide (CO_2) by heating at 900°C for 4 hours in the presence of copper oxide. The CO_2 was then reduced to filamentous graphite in the presence of cobalt at 900°C. The investigators placed the resultant graphite-on-cobalt in the ion source of a spectrometer and analyzed it using methods described previously (Turteltaub et al 1993).

Because AMS measures the ratio of a rare isotope relative to a stable isotope, the investigators measured concentrations of ^{14}C relative to ^{13}C and normalized them to a $^{14}C/^{12}C$ ratio (Vogel et al 1995). The ratios were converted to mass of ^{14}C-benzene based on the specific activity of the benzene after subtracting the natural radiocarbon content contributed from the sample and the tributyrin carrier, when added.

AMS provides a measure of radiocarbon content only and gives no information on the origin or source of the radiocarbon. Thus, all these data were reported as *benzene equivalents*.

Radiocarbon is present in all biological material. Present-day natural levels (background) of ^{14}C in living biological materials are 112 ± 5 attomol (ie, 10^{-18} mol) per g carbon. Based on analysis of more than 500 samples, tributyrin contains 15 ± 2 attomol ^{14}C/mg. For the time-course data in this study, historical data on the ^{14}C content in unexposed animals was used as background. The natural amount of radiocarbon in all biological material is constant in these animals due to their age and growth conditions; generally, the amount varies by less than 10%.

Cumulative Burden

To compare total levels of metabolites in liver and bone marrow, Turteltaub and Mani calculated an area under the curve (AUC) for each metabolite or adduct in each tissue by estimating integrals at different times for up to 48 hours after administering benzene.

RESULTS

HPLC-AMS Detection of Low Levels of Benzene Metabolites

AMS allowed low femtomolar concentrations of ^{14}C-labeled benzene metabolites separated by HPLC to be detected in biological samples (eg, from mouse urine, as shown in Figure 2). The distribution of ^{14}C across the HPLC separation (black bars) generally matched the profile of benzene metabolite standards (hydroquinone, MA, phenyl glucuronide, catechol, hydroquinone sulfate, and phenyl sulfate). Figure 2 also shows a high amount of a rapidly eluting, unidentified product in the sample.

Detection of Dose-Dependent Benzene Metabolites in Tissues of B6C3F$_1$ Mice

The Commentary Table summarizes the major results of the experiments involving B6C3F$_1$ mice. Levels of several benzene metabolites detected in urine one hour after IP injection of benzene were dose dependent over the range 50 ng to 500 mg benzene per kg body weight (Figure 3). At the lowest end of the range (50 to 500 ng benzene per kg body weight), the slope of the dose-response curve was flatter than for higher doses but was still above background levels. In addition, the relative amounts of benzene metabolites detected in urine remained constant over the dose range, 50 ng/kg to 500 mg/kg: unidentified metabolite ≥ phenyl sulfate > MA > phenyl glucuronide ≥ hydroquinone sulfate. The unidentified metabolite, phenyl sulfate, and MA were therefore considered major urinary metabolites of benzene, and phenyl glucuronide and hydroquinone sulfate minor urinary metabolites. The investigators reported that they detected low levels of free catechol and hydroquinone but that levels of these metabolites did not vary with benzene dose.

The pattern of metabolites detected in liver, bone marrow, and plasma differed from that found in urine one hour after IP benzene injection (Figure 4 and Table 1). Levels of MA, hydroquinone, catechol, and phenol were analyzed after injecting 5, 50, or 5000 ng benzene per kg body weight. (The unidentified metabolite detected in urine was not measured in these tissues.) In the liver, levels of MA, catechol, and hydroquinone varied with the benzene dose, but levels of phenol did not. In plasma and bone marrow, no metabolites depended on benzene dose.

Kinetics and Total Levels of Metabolite Formation in B6C3F$_1$ Mouse Tissues

The investigators examined the kinetics of hydroquinone, catechol, phenol, and MA formation and clearance in plasma, liver and bone marrow from 0 to 48 hours after IP injection of 5 µg ^{14}C-benzene per kg body weight

Commentary Table. Dose-Dependent Products of Benzene Metabolism Detected After IP Administration of Benzene to B6C3F$_1$ Mice[a]

Product	Urine[b]	Liver	Plasma	Bone Marrow
Benzene metabolites	phenyl sulfate[c], MA, phenyl glucuronide, HQ sulfate (but not HQ or CAT)	MA[c], HQ, CAT (but not phenol)	None[d]	None[d]
DNA adducts		Yes[e]		Yes[e]
Protein adducts		Yes		Yes[e]

[a] Urine was collected over 24 hours after exposure. Levels of products in liver, plasma, and bone marrow are compared at 1 hour after benzene injection, the only timepoint at which all tissues were compared. Empty cells indicate no testing. HQ = hydroquinone; CAT = catechol.

[b] An unidentified product was detected at higher concentrations than any of the identified metabolites. This product was not looked for in the liver, plasma, or bone marrow.

[c] Phenyl sulfate and MA were the metabolites with the highest concentrations in urine and liver, respectively, one hour after benzene injection. The levels of these metabolites were dose dependent.

[d] MA and MA and HQ were the metabolites with the highest concentrations in plasma and bone marrow, respectively, one hour after benzene injection. The levels of these metabolites were not dose dependent.

[e] Curvilinear dose-response curve with flattening at the lowest end (5–500 ng benzene per kg body weight) of the dose range at 1 and 12 hours after benzene injection.

(Figure 5). Metabolite levels in liver and plasma peaked within 0.5 to 5 hours after injection, but peaks in metabolite levels in bone marrow were not obvious. Turteltaub and Mani also calculated AUCs for each metabolite, which they reported to be highly variable (Table 2). In liver and bone marrow, hydroquinone and MA were the major metabolites (hydroquinone the most prevalent in liver and MA the most prevalent in bone marrow), with phenol and catechol detected at much lower levels. Levels of all metabolites were low in plasma, with hydroquinone having the highest concentration of the metabolites evaluated.

Adduct Detection in B6C3F$_1$ Mice

The investigators measured benzene-derived DNA and protein adducts in B6C3F$_1$ mice 1 and 12 hours after IP injection of 5 ng to 500 mg benzene per kg body weight. Accumulation of DNA and protein adducts was dose dependent at both time points in liver and bone marrow (Figure 6). The level of protein adducts in liver was linear with benzene dose over the range 5 ng to 50 mg/kg, appearing to plateau at higher doses. The dose-response curves for DNA adducts in liver and bone marrow and for protein adducts in bone marrow were curvilinear with a pronounced flattening of the curve at the lowest benzene concentrations (5 to 500 ng/kg).

In liver, levels of both protein and DNA adduct levels tended to be higher 1 hour after dosing than 12 hours after. In bone marrow, adduct levels were either equivalent at both times (protein) or higher after 12 hours (DNA). These data were confirmed by the AUC data (Figure 7).

Kinetics and Total Levels of DNA and Protein Adducts in Rodent Liver and Bone Marrow

The kinetics of adduct accumulation in bone marrow was similar in Fischer rats and in B6C3F$_1$, C57BL/6, and DBA/2 mice (after injection of 5 μg benzene per kg): maximum levels of both adducts were reached within 12 hours of benzene administration (Table 3 and bottom panel of Figure 8). AUCs for protein and DNA adducts in bone marrow varied by rodent group as follows: B6C3F$_1$ > DBA/2 > C57BL/6 > Fischer rat (Table 3). Levels of DNA and protein adducts in livers of B6C3F$_1$, C57BL/6, and DBA/2 mice peaked within 0.5 to 1 hour after benzene administration and declined rapidly thereafter (Table 4 and top panel of Figure 8). In contrast, DNA and protein adduct levels in livers of Fischer rats continued to increase until 6 to 12 hours after exposure before declining. In all animals tested, levels of protein adducts in liver and bone marrow were higher by 10- to 20-fold than levels of DNA adducts. AUCs of both DNA and protein adducts in liver were greater in B6C3F$_1$ mice than in Fischer rats although the pattern of AUC levels for bone marrow across different rodents was not found for liver.

Adduct Formation in Rats and $B6C3F_1$ Mice After Benzene Inhalation

The investigators detected DNA and protein adducts in liver and bone marrow of $B6C3F_1$ mice and rats up to 24 hours after exposure to 5 µg benzene per kg body weight by inhalation (Tables 5 and 6). Adduct levels in mouse liver and bone marrow were consistently higher than those of the rat.

The kinetics of adduct appearance in mouse liver and bone marrow were similar after inhalation and IP exposures; adduct levels peaked 1 and 12 hours after exposure in liver and bone marrow, respectively (Figure 9). In contrast, adduct kinetics in rats differed by exposure route: After IP injection, both protein and DNA adducts increased for up to 6 to 12 hours; after inhalation exposure, adduct formation either peaked within an hour of exposure or showed no clear peak at all (Figure 10). Regardless of the route of exposure or tissue, levels of protein adducts were greater than DNA adduct levels in mice and rats (Tables 3 and 4). Adduct levels in both species were generally lower after inhalation than after IP injection (Figure 11).

DISCUSSION

Turteltaub and Mani have performed an important and innovative study of the dosimetry of benzene metabolism, producing data that address several critical issues. Using a novel and sensitive analytic technique—AMS coupled with HPLC—they were able to detect dose-dependent formation of benzene metabolites, including DNA and protein adducts, in the tissues of mice at levels 100 times lower than any found in previous studies. In both mice and rats they detected higher levels of benzene metabolites in liver and bone marrow than in plasma. These findings indicate that benzene reaches tissues and is metabolized there, even at levels near those to which humans may be exposed in ambient air, 1 to 10 ppb. (By way of comparison with the doses used in the current study, injecting 10 µg benzene per kg body weight is equivalent to a mouse inhaling 2 ppb benzene for 2.5 minutes.)

In addition, adduct levels detected in bone marrow and liver in different rodents generally correlated well with the ability of benzene to induce tumors in that species or strain. This finding is important because it suggests that the formation of adducts may be an early marker of benzene toxicity or carcinogenicity. Turteltaub and Mani also showed that benzene doses over a wide range generate similar patterns of metabolites in $B6C3F_1$ mice, suggesting that the pattern of benzene metabolism is similar at widely disparate concentrations. This outcome differs from those reported in previous rodent, human, and nonhuman primate studies in which different patterns of metabolites were found at different exposure concentrations (Sabourin et al 1992; Henderson 1996; Mathews et al 1998; Rothman et al 1998). The reasons for these differences in results among studies are not clear. In the current study, only low levels of hydroquinone were detected in the urine of exposed mice, exclusively in the sulfate conjugate form. By contrast, earlier studies indicated that in mice, hydroquinone glucuronide was a major urinary metabolite of benzene—comprising up to 40% of all urinary metabolites—especially at low doses of benzene (Sabourin et al 1989; Henderson 1996; Mathews et al 1998). The absence of hydroquinone glucuronide in the current study, which most likely resulted from the investigators not including an inhibitor of the enzyme glucuronidase in samples to prevent glucuronide breakdown, makes the results difficult to compare with previous rodent studies (Henderson et al 1996; Mathews et al 1998). Moreover, these rodent studies and studies of urinary benzene metabolites in humans and nonhuman primates found that the change in proportion of hydroquinone glucuronide relative to other metabolites at different levels of benzene exposure was the main indicator of a dose-dependent shift in metabolism (Sabourin et al 1992; Henderson et al 1996; Mathews et al 1998; Rothman et al 1998). Turteltaub and Mani's inability to detect a dose-dependent shift in urinary metabolites in the current study may be due, in part, to an inability to detect this major metabolite of benzene.

The investigators' use of an AUC approach provided a more accurate depiction of metabolite accumulation in tissues over time than data collected at a single time point. Calculation of AUCs produced numbers with large variances. Mani and Turteltaub speculate that these variances may have been due to variation among the animals, investigator error in multiple sampling, or artifacts related to measurement of HPLC fractions.

In addition, the finding that more benzene metabolites were formed after IP injection compared with inhalation of the same calculated dose may be important because it suggests that exposure via different routes may differ quantitatively. Such differences in metabolite formation after IP exposure to benzene compared with oral exposure have been described previously (Sabourin et al 1989). However, although Turteltaub and Mani calculated the inhaled dose to equal the injected dose, previous estimates suggest that the actual dose absorbed after inhalation is probably only approximately one-half the inhaled dose (Sabourin et al 1987; Travis et al 1990). This discrepancy may account for the difference in metabolite levels detected after exposure via inhalation versus IP injection.

Turteltaub and Mani's finding that the formation of DNA and protein adducts in liver and bone marrow depended on benzene dose suggests that adduct levels may be biomarkers of benzene exposure. The usefulness of benzene adducts as biomarkers has been evaluated in other studies. Recent data suggest that plasma levels of the hemoglobin and albumin adducts of benzene oxide, which are protein adducts of a benzene metabolite, may be useful as markers of occupational-level exposure to benzene (Yeowell-O'Connell et al 1998), but no information is available at exposure doses comparable to those used in the current study. Turteltaub and Mani did not identify the adducts they detected, so they do not know if the same adducts are formed in both liver and bone marrow. Identification of these adducts is critical for determining their utility as biomarkers of benzene exposure. In addition, Turteltaub and Mani evaluated adduct formation in liver and bone marrow only, which are sites that cannot be routinely sampled. Further quantification and characterization of DNA and protein adducts in urine and blood is needed for their validation as useful biomarkers of benzene exposure.

The findings of this study require a few caveats. Although the results show the sensitivity of AMS coupled with HPLC, they also illustrate potential drawbacks. First, in the current study, urine of B6C3F$_1$ mice exposed to ^{14}C-benzene contained a large peak of radioactivity that could not be identified by HPLC. The investigators did not look for this material in plasma or bone marrow; thus, the peak might also have been present in samples from these tissues, with an uncertain impact on the results. This unidentified radioactive material may be a contaminant of the radioactive material used in the assays, or as Turteltaub and Mani speculate, a previously unidentified metabolite or a decomposition product of a known benzene metabolite. Second, detection by AMS requires administration of radiolabeled benzene to study animals. Although this approach uses only extremely low levels of reagents, it is not broadly applicable to controlled exposure studies in humans because benzene is classified as a known human carcinogen. Third, although the AMS approach may be inherently more analytically sensitive than alternative approaches, the failure to include glucuronidase inhibitors in samples in the current study makes comparisons of some sets of results difficult to relate to the results of others.

Turteltaub and Mani's report of flattening of the dose-response curves for urinary metabolites and adduct formation at the lowest end of the benzene exposure range (5 to 500 ng/kg) suggests that metabolism of benzene to activated metabolites occurs even at these very low doses. Because this dose-response pattern suggests no obvious threshold for benzene effects, this finding may have important ramifications for understanding the human response to benzene at doses close to ambient levels. However, interpretation of these results is difficult. First, many of the radioactivity values reported for low benzene exposures, particularly those shown in Figure 6, are close to background. Interpretation of whether these values are above background is difficult because there are uncertainties about how the ^{14}C content was determined and corrected in the measured values. Turteltaub and Mani state that a background level of ^{14}C was subtracted from the measured values, but they do not give the number they used. The natural background level is thought to be approximately 8 pg benzene equivalents per g tissue, which is similar to levels detected at the lowest end of the dose response in this study. Second, the data in this study are presented using log-log scales. This method may not be the most appropriate for data at the lowest end of the range; other transformations might show a different dose-response pattern.

SUMMARY AND CONCLUSIONS

In this innovative study, the investigators showed that the sensitive technique of HPLC coupled with AMS could detect dose-dependent formation of many benzene metabolites in the liver, bone marrow, and urine of rodents. Dose-dependent formation of DNA and protein adducts in liver and bone marrow was detected over a wide dose range, including very low exposure. These findings indicate that benzene reaches target tissues and is metabolized there, even at levels near those in ambient air to which humans may be exposed. Levels of adducts detected in bone marrow and liver in different rodents generally correlated well with the ability of benzene to induce tumors in that species or strain, suggesting that the formation of these adducts may be an early marker of benzene toxicity or carcinogenicity. The results also suggest, but do not show conclusively, that the dose-response curve for benzene in mice lacks an obvious threshold at the lowest exposure levels evaluated. Further studies are required to resolve the shape of the dose-response curve for humans at these low benzene levels.

ACKNOWLEDGMENTS

The Health Review Committee thanks the ad hoc reviewers for their help in evaluating the scientific merit of the Investigators' Report. The Committee is also grateful to Dr Geoffrey Sunshine for his assistance in preparing its Commentary, and to Sally Edwards, Jenny Lamont, and Fred Howe for their roles in publishing this Research Report.

REFERENCES

American Conference of Government and Industrial Hygienists. 1999. Guide to Occupational Exposure Values. ACGIH, Cincinnati OH.

Bechtold WE, Sabourin PJ, Henderson RF. 1988. A reverse isotope dilution method for determining benzene and metabolites in tissues. J Anal Toxicol 12:176–179.

Chen H, Eastmond DA. 1995. Topoisomerase inhibition by phenolic metabolites: A potential mechanism for benzene's clastogenic effects. Carcinogenesis 16:2301–2307.

Creek MR, Mani C, Vogel JS, Turteltaub KW. 1997. Tissue distribution and macromolecular binding of extremely low doses of [^{14}C]-benzene in $B_6C_3F_1$ mice. Carcinogenesis 18:2421–2427.

Eastmond DA, Schuler M, Frantz C, Chen H, Parks R, Wang L, Hasegawa L. 2001. Characterization and Mechanisms of Chromosomal Alterations Induced by Benzene in Mice and Humans. Research Report 103. Health Effects Institute, Cambridge MA.

Eastmond DA, Smith DT, Irons RD. 1987. An interaction of benzene metabolites reproduces the myelotoxicity observed with benzene exposure. Toxicol Appl Pharmacol 91:85–95.

Environmental Protection Agency (US). 1998a. Carcinogenic Effects of Benzene: An Update. EPA/600P-97/001F. Office of Research and Development, Washington DC.

Environmental Protection Agency (US). 1998b. Toxicological Review of Benzene (noncancer effects) [preliminary draft]. USEPA NCEA-S-0455. EPA National Center for Environmental Assessment, Washington DC. (Available on the Web at *cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=12160*.)

Goldstein BD, Witz G. 2000. Benzene. In: Environmental Toxicants: Human Exposures and Their Health Effects, 2nd ed (Lippmann M, ed), pp 121–149. John Wiley & Sons, New York NY.

Henderson RF. 1996. Species difference in the metabolism of benzene. Environ Health Perspect 104(Suppl 6):1173–1175.

Ho TY, Witz G. 1997. Increased gene expression in human promyeloid leukemia cells exposed to *trans,trans*-muconaldehyde, a hematotoxic benzene metabolite. Carcinogenesis 18:739–744.

Huff JE, Haseman JK, DeMarini DM, Eustis S, Maronpot RR, Peters AC, Persing RL, Chrisp CE, Jacobs AC. 1989. Multiple-site carcinogenicity of benzene in Fischer 344 rats and $B6C3F_1$ mice. Environ Health Perspect 82:125–163.

Huff J. 1993. National Toxicology Program Technical Report on the Toxicology and Carcinogenesis Studies of Benzene (CAS No. 71-43-2) in F344/N Rats and $B6C3F_1$ Mice (gavage studies). National Institutes of Environmental Health Sciences, Research Triangle Park NC.

International Agency for Research on Cancer. 1982. Benzene. In: Some Industrial Chemicals and Dyestuffs, pp 93–148. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol 29. IARC, Lyon, France.

Longacre LS, Kocsis JJ, Snyder R. 1981. Influence of strain differences in mice on the metabolism and toxicity of benzene. Toxicol Appl Pharmacol 60:398–409.

Mathews JM, Etheridge AS, Matthews HB. 1998. Dose-dependent metabolism of benzene in hamsters, rats, and mice. Toxicol Sci 44:14–21.

Occupational Safety and Health Administration (US). 1987. Final rule on occupational exposure to benzene. Fed Regist 54:34660–34762.

Rothman N, Bechtold WE, Yin S-N, Dosemeci M, Li G-L, Wang Y-Z, Griffith WC, Smith MT, Hayes RB. 1998. Urinary excretion of phenol, catechol, hydroquinone, and muconic acid by workers occupationally exposed to benzene. Occup Environ Med 55:705–711.

Sabourin PJ, Bechtold WE, Birnbaum LS, Lucier G, Henderson RF. 1988a. Differences in the metabolism and disposition of inhaled [^{3}H] benzene by F344/N rats and $B6C3F_1$ mice. Toxicol Appl Pharmacol 94:128–140.

Sabourin PJ, Bechtold WE, Griffith WC, Birnbaum LS, Lucier G, Henderson RF. 1989. Effect of exposure concentration, exposure rate and route of administration on metabolism of benzene by F344 rats and $B6C3F_1$ mice. Toxicol Appl Pharmacol 99:421–444.

Sabourin PJ, Bechtold WE, Henderson RF. 1988b. A high pressure liquid chromatographic method for the separation and quantitation of water-soluble radiolabeled benzene metabolites. Anal Biochem 170:316–327.

Sabourin PJ, Chen BT, Lucier G, Birnbaum LS, Fisher E, Henderson RF. 1987. Effect of dose on the absorption and excretion of [^{14}C] benzene administered orally or by inhalation in rats and mice. Toxicol Appl Pharmacol 87:325–336.

Sabourin PJ, Muggenburg BA, Couch RC, Lefler D, Lucier G, Birnbaum LS, Henderson RF. 1992. Metabolism of [^{14}C]benzene by cynomolgus monkeys and chimpanzees. Toxicol Appl Pharmacol 114:277–284.

Smith MT. 1996. The mechanism of benzene-induced leukemia: A hypothesis and speculations on the causes of leukemia. Environ Health Perspect 104(Suppl 6):1219–1225.

Snyder R, Hedli CC. 1996. An overview of benzene metabolism. Environ Health Perspect 104(Suppl 6):1165–1171.

Travis CC, Quillen JL, Arms AD. 1990. Pharmacokinetics of benzene. Toxicol Appl Pharmacol 102:400–420.

Turteltaub KW, Vogel JS, Frantz CE, Fultz E. 1993. Studies on DNA adduction with heterocyclic amines by accelerator mass spectrometry: A new technique for tracing isotope-labeled DNA adduction. In: Postlabeling Methods for Detection of DNA Adducts (Phillips DH, Castegnaro M, Bartsch H, eds), pp 293–300. International Agency for Research on Cancer, Lyon, France.

Vogel JS. 1992. Rapid production of graphite without contamination for biological AMS. Radiocarbon 34:344–350.

Vogel JS, Turteltaub KW, Finkel R, Nelson DE. 1995. Accelerator mass spectrometry: Isotope identification at attomole sensitivity. Anal Chem 67:353A–359A.

Wallace L. 1996. Environmental exposure to benzene: An update. Environ Health Perspect 104(Suppl 6):1129–1136.

Witz G, Maniara WM, Mylavarapu VJ, Goldstein BD. 1990. Comparative metabolism of benzene and *trans, trans*-muconaldehyde to *trans, trans*-muconic acid in DBA/2 and C57BL/6 mice. Biochem Pharmacol 40:1275-1280.

Witz G, Zhang Z, Goldstein BD. 1996. Reactive ring-opened aldehyde metabolites in benzene hematotoxicity. Environ Health Perspect 104(Suppl 6):1195–1199.

Yeowell-O'Connell K, Rothman N, Smith MT, Hayes RB, Li G, Waidyanatha S, Dosemeci M, Zhang L, Yin S, Titenko-Holland N, Rappaport SM. 1998. Hemoglobin and albumin adducts of benzene oxide among workers exposed to high levels of benzene. Carcinogenesis 19:1565–1571.

RELATED HEI PUBLICATIONS: AIR TOXICS

Report Number	Title	Principal Investigator	Publication Date
HEI Research Reports			
	Biomarkers in Czech Workers Exposed to 1,3-Butadiene: A Transitional Epidemiologic Study (in press)	RJ Albertini	2003
	Validation of Biomarkers in Humans Exposed to Benzene (in press)	Q Qu	2003
108	Case-Cohort Study of Styrene Exposure and Ischemic Heart Disease	GM Matanoski	2002
107	Emissions from Diesel and Gasoline Engines Measured in Highway Tunnels		
	Real-World Particulate Matter and Gaseous Emissions from Motor Vehicles in a Highway Tunnel	AW Gertler	2002
	Airborne Carbonyls from Motor Vehicle Emissions in Two Highway Tunnels	D Grosjean	2002
103	Characterization and Mechanisms of Chromosomal Alterations Induced by Benzene in Mice and Humans	DA Eastmond	2001
101	Respiratory Epithelial Penetration and Clearance of Particle-Borne Benzo[*a*]pyrene	P Gerde	2001
92	1,3-Butadiene: Cancer, Mutations, and Adducts		2000
	Part I: Carcinogenicity of 1,2,3,4-Diepoxybutane	RF Henderson	
	Part II: Roles of Two Metabolites of 1,3-Butadiene in Mediating Its in Vivo Genotoxicity	L Recio	
	Part III: In Vivo Mutation of the Endogenous *hprt* Genes of Mice and Rats by 1,3-Butadiene and Its Metabolites	VE Walker	
	Part IV: Molecular Dosimetry of 1,3-Butadiene	IA Blair	
	Part V: Hemoglobin Adducts as Biomarkers of 1,3-Butadiene Exposure and Metabolism	JA Swenberg	
87	Development of Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry Methods for Determination of Urinary Metabolites of Benzene in Humans	AA Melikian	1999
67	Development of Methods for Measuring Biological Markers of Formaldehyde Exposure	TR Fennell	1994

Continued

Copies of these reports can be obtained from the Health Effects Institute and many are available at www.healtheffects.org.

RELATED HEI PUBLICATIONS: AIR TOXICS

Report Number	Title	Principal Investigator	Publication Date
53	Use of Physical Chemistry and in Vivo Exposure to Investigate the Toxicity of Formaldehyde Bound to Carbonaceous Particles in the Murine Lung	GJ Jakab	1992
51	Effects of Formaldehyde on Xenotransplanted Human Respiratory Epithelium	AJP Klein-Szanto	1992
49	Mechanisms of Aldehyde-Induced Bronchial Reactivity: Role of Airway Epithelium	GD Leikauf	1992
HEI Communications			
6	Partnership to Examine Emerging Health Effects: EC/HEI Workshop on 1,3-Butadiene		1999
2	Research Priorities for Mobile Air Toxics		1993
HEI Program Summaries			
	Research on Air Toxics		1999

Copies of these reports can be obtained from the Health Effects Institute and many are available at www.healtheffects.org.

HEI

HEALTH
EFFECTS
INSTITUTE

Charlestown Navy Yard
120 Second Avenue
Boston MA 02129-4533 USA
+1-617-886-9330
www.healtheffects.org

RESEARCH
REPORT

Number 113
February 2003