RESEARCH REPORT

HEALTH EFFECTS INSTITUTE

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1,3-Butadiene: Cancer, Mutations, and Adducts

Includes Commentaries by the Institute's Health Review Committee

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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 100 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 US. 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 92

Cancer, Mutations, and Adducts in Rats and Mice Exposed to Butadiene and Its Metabolites

BACKGROUND

1,3-Butadiene (BD) is an occupational and environmental pollutant that is widely used in the manufacture of resins, plastics, and synthetic rubber; it is also found in combustion emissions from motor vehicles, stationary sources, and cigarette smoke. Ambient exposures to BD (0.3 to 10 parts per billion [ppb]) are orders of magnitude lower than those that occur in occupational settings (10 to 300,000 ppb), but they are a public health concern because BD may be a human carcinogen. In the Clean Air Act Amendments of 1990, BD is listed as a hazardous air pollutant and a mobile-source toxic air pollutant. Moreover, worldwide regulatory interest has evolved in the potential health effects of occupational and ambient exposures to BD.

Epidemiologic studies have suggested that workers occupationally exposed to BD have an increased incidence of cancers of the lymphatic system and of those organs and systems in the body that produce blood cells. Interpretation of these studies has been controversial, however, because of inconsistencies among the results and because some workers may have been exposed to other chemicals in addition to BD. In situations of such uncertainty, data from animal studies are often used to fill information gaps about humans by extrapolating findings from laboratory studies in which animals have been exposed to high doses of a chemical to situations in which human subjects have been exposed to low doses. Consequently, researchers have produced a large range of human cancer risk estimates for BD depending on which animal species and which data they used in their extrapolation models.

One question is: Which species *is* the best model to use for assessing the human risk of cancer from exposure to BD? Butadiene is known to cause tumors in many organs in rats and mice. These two species differ markedly, however, in their carcinogenic response to BD in that mice have been found to be about 100 times more sensitive than rats to BD exposure. Some evidence indicates this difference may relate to how rats and mice metabolize BD and convert it either to reactive (and possibly carcinogenic) intermediates or to inactive forms that are excreted from the body. Whether humans metabolize BD more like mice or more like rats is uncertain, as is where the human response to BD exposure fits in the range of rodent sensitivity to BD's cancer-causing effects.

The Health Effects Institute's air toxics research program to reduce uncertainties in estimating the human health risks associated with exposure to BD includes (1) studies of the reactivity of BD and its metabolites in animals and people and (2) the development of methods to measure biomarkers (indicators of exposure or of early biological effects of exposure) to use in cross-species studies and in studies of human populations. Five independent studies from the initial phase of HEI's BD research program are summarized in this Statement and are presented in detail in this Research Report.

APPROACH

When HEI's program was initiated, scientists knew that BD itself is not carcinogenic. Rather, BD is transformed to reactive metabolites that can bind to DNA (forming adducts), thus causing genetic mutations and possibly initiating the carcinogenic response. The role of individual metabolites in BD-induced carcinogenesis, however, was not known. Furthermore, the metabolites exist in more than one stereochemical (or three-dimensional) form. Because enzymes may react preferentially with a specific form, these stereochemical configurations may be important in species sensitivity. Some of the products from BD reacting with cellular DNA or proteins had been identified and considered for use as biomarkers of exposure or of a biologically effective dose. However, sensitive analytical methods needed to be developed and validated if these biomarkers were to be useful in animal or human studies.

This Statement, prepared by the Health Effects Institute, is a summary of five research projects conducted by Drs. Ian Blair (University of Pennsylvania, Philadelphia), Rogene Henderson (Lovelace Respiratory Research Institute, Albuquerque, NM), Leslie Recio (Chemical Industry Institute of Toxicology, Research Triangle Park, NC), James Swenberg (University of North Carolina at Chapel Hill), and Vernon Walker (New York State Department of Health, Albany). The following Research Report contains the five detailed Investigators' Reports and three Commentaries on the studies prepared by the Institute's Health Review Committee.

The studies reported here were designed to advance our understanding of the roles of different metabolites in BD-induced carcinogenesis and of the differences in sensitivity among species, and to develop methods for identifying and measuring biomarkers. The investigators focused on two BD metabolites (1,2-epoxy-3-butene [BDO] and 1,2,3,4-diepoxybutane [BDO_2]) that researchers had suspected may play a role in BD carcinogenesis; they also developed information on other metabolites that may be important but had not been extensively studied.

Dr. Rogene Henderson (Lovelace Respiratory Research Institute) exposed mice and rats to BDO_2 to determine whether these species differ in their carcinogenic response to this metabolite. Dr. Leslie Recio (Chemical Industry Institute of Toxicology) and Dr. Vernon Walker (New York State Department of Health) compared the mutagenicity of BD, BDO, and BDO_2 in mice and rats. Dr. Ian Blair (University of Pennsylvania) developed methods for measuring DNA adducts derived from BD metabolites in the tissues and urine of rats and mice with the goal of comparing the levels of adducts in the two species and identifying possible biomarkers. Dr. James Swenberg (University of North Carolina at Chapel Hill) developed a sensitive method for detecting adducts formed between BD metabolites and a blood protein (hemoglobin) and measured these adducts in animals and humans exposed to BD. The investigators shared tissues from animals that were exposed by inhalation to BD or its metabolites at either the Chemical Industry Institute of Toxicology or Lovelace Respiratory Research Institute and, in some cases, developed collaborative ventures.

RESULTS AND IMPLICATIONS

These five studies have advanced our understanding of BD metabolism, genotoxicity, and carcinogenicity. Some of the most noteworthy accomplishments were

- direct demonstration that BDO_2 is carcinogenic in both rats and mice;
- corroboration of earlier findings that BD and its metabolite BDO have greater mutagenic potencies and form more adducts in mice than in rats, which agrees with the different sensitivities the two species exhibit in BD-induced carcinogenesis; however, one study found that BDO_2 was more mutagenic in rats than in mice;
- demonstration that each BD metabolite tested produces a characteristic pattern of mutations in rodent DNA; these patterns show potential as biomarkers for human exposure to BD; and
- development of sensitive and specific methods for measuring biomarkers of BD exposure (hemoglobin and DNA adducts derived from BD metabolites) and preliminary validation of using hemoglobin adducts as biomarkers in animals and humans.

The investigators successfully answered many of the questions they set out to address; some unexpected results will require further research to understand the range of their implications. Some avenues of needed research include

- the roles of specific BD metabolites (including their various stereochemical forms) in the carcinogenicity, genotoxicity, and cytotoxicity of BD;
- the relative importance of point mutations and large genetic deletions in BD carcinogenicity, and the forms of the promutagenic lesions;
- the dose-response relations among BD exposure concentrations and the levels of biomarkers (especially the limits of detection relative to ambient exposure conditions and the sources of background or endogenous adducts); and
- if or how the different biomarkers are indicators of cancer risk.

Some of these questions are being addressed in an HEI-supported study led by Dr. Richard Albertini, which is being conducted in the Czech Republic. The goal of that effort is to determine whether a dose-response relation can be established between the level of an individual's exposure and the amount of various biomarkers detected in blood or urine samples from workers exposed to BD.

HEALTH EFFECTS INSTITUTE

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PREFACE TO *1,3-BUTADIENE: CANCER, MUTATIONS, AND ADDUCTS*

1,3-Butadiene (BD)* is a four-carbon gaseous chemical used in the manufacture of resins, plastics, and synthetic rubber. It is also a product of combustion and is present in emissions from motor vehicles, stationary sources, and cigarettes. Although the highest BD exposures occur in industrial settings (less than 10 to 370 ppm), ambient exposures (which range from less than 1 ppb to 10 ppb) may be a public health concern because BD is carcinogenic in rats and mice and may be a human carcinogen (reviewed in Melnick and Kohn 1995; European Centre for Ecotoxicity and Toxicology of Chemicals 1997; Himmelstein et al. 1997; International Agency for Research on Cancer [IARC] 1999).

In 1994, the Health Effects Institute initiated an air toxics research program to address the health risks of ambient exposures to toxic air pollutants, including BD. The overall goal of this program was to acquire information that would reduce uncertainties in evaluating the human health risks associated with exposure to low levels of toxic air pollutants from mobile sources. This Preface (1) presents the regulatory context for this work, (2) briefly summarizes the evidence for BD carcinogenicity, (3) describes the goals of HEI's BD research program, which included the five studies presented in this Research Report (Henderson, Recio, Walker, Blair, and Swenberg), and (4) outlines the procedures HEI used to develop and manage its BD research program and to review the results of these studies.

REGULATORY BACKGROUND

Butadiene is referenced in sections of the Clean Air Act Amendments of 1990 dealing with hazardous air pollutants and mobile-source toxic air pollutants. Section 112 lists BD as one of the 188 chemicals designated as hazardous. This section also requires that the U.S. Environmental Protection Agency (EPA) develop a national strategy to control emissions of hazardous air pollutants from urban sources. Under this requirement, the Agency had to identify at least 30 hazardous air pollutants that account for 90% of the aggregate emissions from area sources and develop regulations for their control. In addition, the Act requires a strategy to reduce by 75% the cancer risk attributed to hazardous air pollutants. Of the 188 pollutants designated as hazardous, five (acetaldehyde, benzene, BD, formaldehyde, and polycyclic organic matter) are also listed as mobile-source toxic air pollutants in Sections 202 and 211 of the Clean Air Act Amendments. In response to lawsuits filed by the Sierra Club, the EPA combined several regulatory efforts that are required by the Clean Air Act into an Integrated Urban Air Toxics Strategy (U.S. EPA 1999). This strategy addresses toxic emissions from all outdoor sources, including stationary, area, and mobile sources. It includes a list of 33 priority hazardous air pollutants that are believed to pose the greatest risk to human health, outlines regulatory plans for reducing emissions of mobile-source air toxics, and suggests actions for developing better information on health risks. Butadiene is one of the 33 high-priority pollutants.

SCIENTIFIC BACKGROUND

Until the 1980s, scientists considered BD to be relatively nontoxic. Early studies of BD toxicity focused on the irritant effects of short-term, high-dose exposures in the workplace where the permissible exposure limit was set at 1,000 ppm as an eight-hour time-weighted average (Occupational Safety and Health Administration 1990). However, evidence of BD's ability to cause cancer in laboratory animals raised concern about its potential as a human carcinogen. The early animal studies showed that inhalation of 1,000 ppm BD produced tumors in several organs in Sprague-Dawley rats (International Institute of Synthetic Rubber Producers 1981). Later studies showed that mice were much more sensitive to BD exposure than rats (National Toxicology Program [NTP] 1984, 1992): Inhalation of 625 ppm BD produced tumors in several organs of mice after 13 weeks of exposure, and exposure levels of 20 ppm and 6.25 ppm produced rare malignant tumors of the heart and lung, respectively. For researchers trying to estimate cancer risks for humans, this difference in sensitivity raises the question of which species most closely resembles humans in its response to BD.

Epidemiologic studies indicate that occupational exposure of workers to BD is associated with an increased incidence of two types of lymphohematopoietic cancers: those of the lymphatic system and those of the organs and systems in the body that produce blood cells (Himmelstein et al. 1997; U.S. EPA1998b; IARC 1999). Possible increases in these two types of cancer were noted in workers exposed to BD in two styrene-BD rubber plants (Meinhardt et al. 1982; Matanoski and Schwartz 1987; Matanoski et al. 1990). More recent and comprehensive studies of the same group have indicated an increased risk of leukemia (but

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

not other types of cancers) in workers with a long duration of employment in the styrene-BD rubber industry (Delzell et al. 1996; Macaluso et al. 1996; Sathiakumar et al. 1998). The interpretation of these studies is somewhat controversial: Even though BD was the major chemical to which these workers had been exposed and the statistical analyses controlled for exposure to styrene, because the subjects had been exposed to several chemicals it is not clear whether the excess cancers were due to BD or other chemicals present in the work place. In addition, studies of the BD monomer industry, where occupational exposure is only to BD, did not show any relation between leukemia and BD exposure. Instead, they reported a small increase in the number of deaths from two forms of sarcoma (tumors that usually arise from connective tissue), lymphosarcoma and reticulosarcoma, in workers who started their employment before World War II (Divine 1990; Divine et al. 1993; Divine and Hartman 1996). These cohort studies have been difficult to interpret (U.S. EPA 1998a; IARC 1999) because of (1) the differences in the types of cancers reported for the BD monomer and the styrene-BD rubber industrial groups, (2) possible confounding exposures of styrene-BD workers to other chemicals, (3) changes in the definition and classification of the lymphohematopoietic cancers, and (4) uncertainty about the relative importance of short-term, high-level exposures versus long-term, low-level exposures.

Because of the limitations of epidemiologic data, quantitative risk assessments of exposure to BD have frequently relied on laboratory animal studies. However, the laboratory studies of rodents present their own challenges for data interpretation because of the marked differences in the sensitivity of rats and mice to the carcinogenic effects of BD. This difference has led researchers to explore mechanisms of the cancer response in the two species, including efforts to determine the sequence of metabolic reactions that initiate cancer and to identify the critical reactions that would explain the species' differences in susceptibility. Once the reasons for the species differences are better understood, human sensitivity to BD exposure can be explored within the context of the rodent studies.

When BD is metabolized, it is either converted to reactive intermediates (which are potentially carcinogenic) or detoxified to water-soluble metabolites (which can be easily released from the body). In all species that have been studied, a common metabolic pathway has been identified (Figure 1): BD is first metabolized by cytochrome P450–monooxygenase to a reactive monoepoxide intermediate, 1,2-epoxy-3-butene (BDO); BDO then can be metabolized further to either 1,2,3,4-diepoxybutane (BDO_2), another

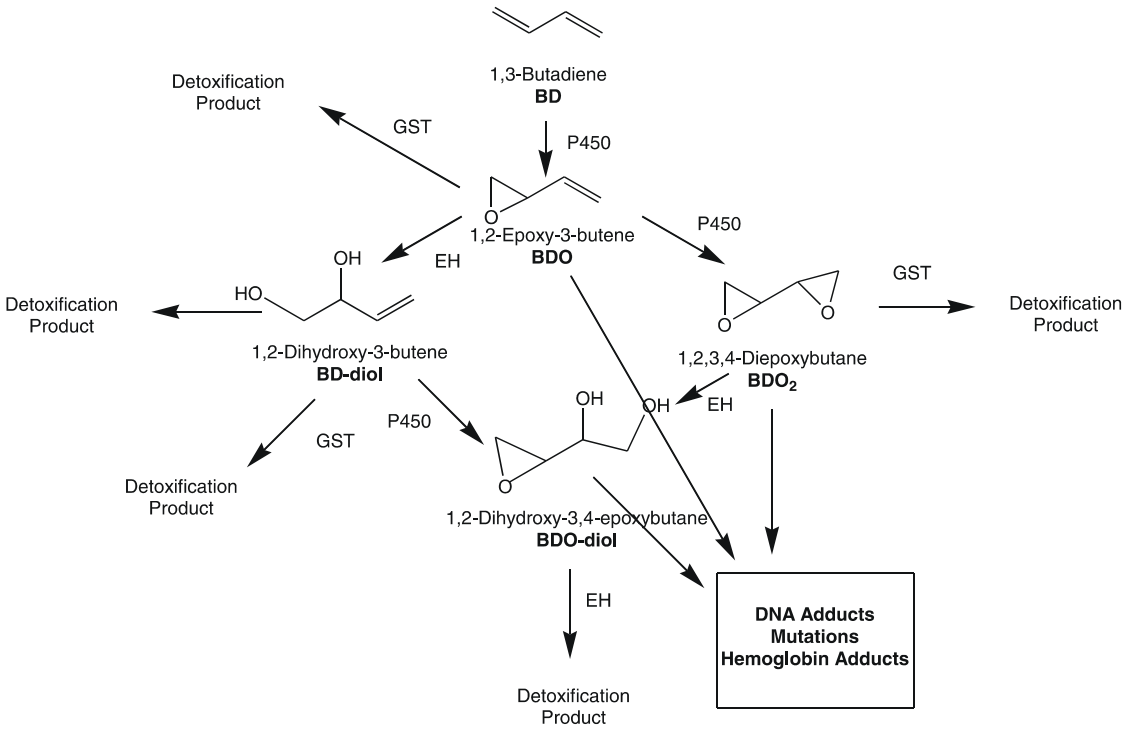

Figure 1. Metabolism of BD. GST = glutathione *S*–transferase, P450 = cytochrome P450, and EH = epoxide hydroxylase; all are enzymes involved in BD metabolism.

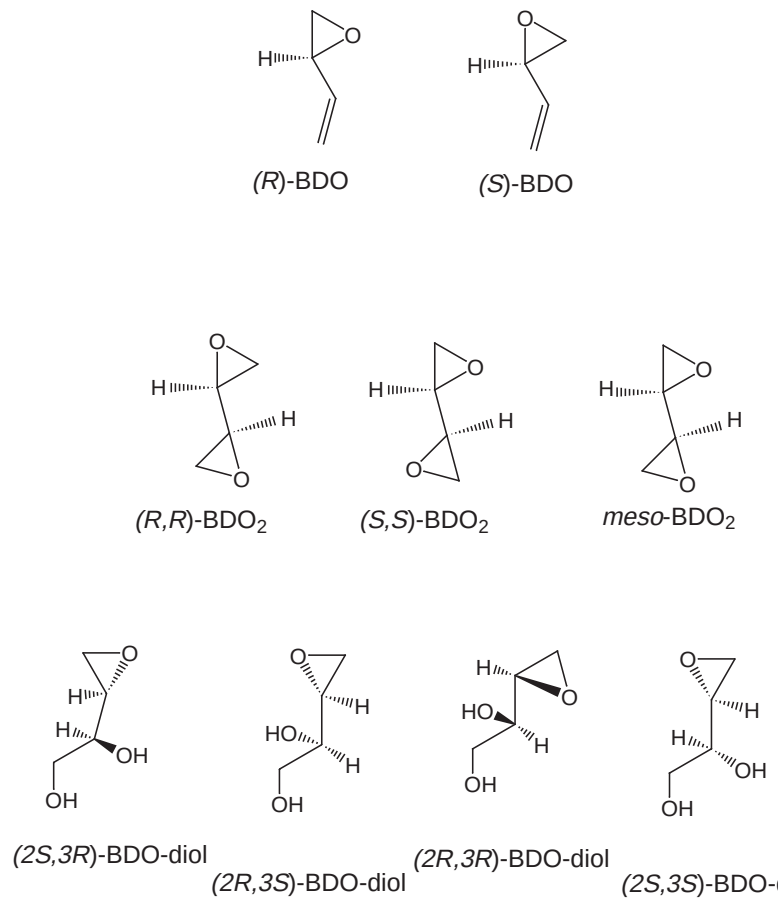

Figure 2. **Stereochemistry of BD.** In some molecules, the asymmetric bonding of some atoms to a carbon atom causes "left hand" (*S*) and "right hand" (*R*) forms (called stereoisomers) to exist. These forms with asymmetric centers, which are considered mirror images, are chemically identical but differ in their three-dimensional configuration. Oxidation of BD to BDO produces one asymmetric center, hence (*R*)- and (*S*)-BDO. Further oxidation converts (*R*)- and (*S*)-BDO to three forms of BDO_2. [The designations (*R,R*) and (*S,S*) indicate two asymmetric centers; the *meso* form also has two asymmetric centers, but their spatial configurations yield overall symmetry within the molecule.] Hydration of the three BDO_2 forms can produce four forms of BDO-diol. Thus, nine possible stereochemical configurations exist for the three BD epoxides. (Adapted with permission from Nieusma et al. 1998.)

reactive metabolite, or to 1,2-dihydroxy-3-butene (BD-diol); BD-diol and BDO_2 can both be oxidized to another epoxide, 1,2-dihydroxy-3,4-epoxybutane (BDO-diol). BDO, BDO_2, and BDO-diol can (1) react with macromolecules such as DNA or proteins, or (2) be detoxified by glutathione *S*–transferase (with the glutathione conjugate being excreted in the urine).

Although BD is converted to the same metabolites in rats and mice, important quantitative differences between the species have been noted in the activities and fate of BD in the body: that is, how BD and its metabolites are absorbed, distributed, transformed, and excreted over time, and in what biological processes they participate while they are present. Research with rats and mice has been directed at (1) identifying which BD metabolites contribute to the cancer process; (2) quantifying the proportion of each metabolite's involvement; and (3) calculating whether interspecies differences in the rates at which the epoxide metabolites are produced and detoxified contribute to the observed differences in the cancer response between the two species (Himmelstein et al. 1997; Richardson et al. 1999). Such information, together with data on the levels of BD intermediates formed in human tissues, would be helpful in identifying the most appropriate animal model for predicting human cancer risks.

Understanding BD metabolism is further complicated by the fact that, as shown in Figure 2, many of its metabolites exist in more than one stereochemical form. (Stereoisomers are compounds that have identical chemical composition but differ from each other in their three-dimensional configuration.) These differences may be important because enzymatic reactions may be stereospecific, which means an enzyme may preferentially act on a specific isomeric configuration. Thus, BD's carcinogenicity may be dependent

on the stereochemical preferences of the enzymes that react with BDO, BDO_2, and BDO-diol. In fact, some studies indicate that different species do demonstrate stereoselectivity for the oxidation and conjugation steps in metabolsm of BD, as well as for toxicity (Krause and Elfarra 1997; Nieusma et al. 1997, 1998).

RISK ASSESSMENT CHALLENGES

Efforts to estimate the cancer risks in humans posed by low-level exposure to BD have been controversial because of limitations in the available exposure information, inconsistencies in the epidemiologic data, and the marked interspecies differences observed in rodent bioassays for cancer. BD is generally accepted to be a carcinogen in animals; the controversy about its being a carcinogen in humans centers around interpretation of the existing epidemiologic data, uncertainty as to which animal data are most suitable for extrapolation to humans, and the calculations of quantitative estimates of risk from low-level ambient exposures.

In 1992, IARC classified BD as a "probable human carcinogen" (Class 2A) on the basis of "limited evidence" in humans and "sufficient evidence" in animals. In 1999, IARC reevaluated BD and concluded that "sufficient evidence" was available for the carcinogenicity of BD and BDO_2 in laboratory animals. However, IARC maintained that the evidence available for human carcinogenicity was still "limited" because it was based on the excess leukemias reported in one large study, and "consistency of results among two or more studies of adequate statistical power" had not yet been reported. Therefore, IARC retained the "probable human carcinogen" designation for BD (IARC 1999).

In its *Eighth Report on Carcinogens*, the NTP (1998a) came to a similar conclusion; namely that evidence for the carcinogenicity of BD in laboratory animals was sufficient, but epidemiologic data in humans were inadequate. Therefore, the NTP classification was "reasonably anticipated to be a human carcinogen." However, as announced in the Federal Register, the NTP (1998b) is upgrading its designation to a "known human carcinogen" in its upcoming *Ninth Report on Carcinogens*.

The EPA also is revising its health risk assessment of BD. Whereas the Agency classified BD as a "probable human carcinogen" in 1985 (U.S. EPA 1985), in its more recent draft risk assessment the staff proposed that BD be classified as a "known human carcinogen" on the basis of the overall evidence from human, animal, and mutagenicity studies (U.S. EPA 1998b). The Environmental Health Committee of the EPA's Scientific Advisory Board, which reviewed the draft document, cited inconsistencies in the epidemiologic data and recommended that the EPA retain the "probable human carcinogen" classification. As of December 1999, the EPA had not made a final decision regarding the BD classification. In its draft document (EPA 1998b), the Agency also reported a quantitative cancer risk assessment of BD on the basis of a linear extrapolation of the increased leukemia risks observed in occupationally exposed workers in the styrene-BD rubber industry and retrospective exposure estimates developed from work history records (Delzell et al. 1995). The EPA's best estimate of human lifetime extra cancer risk (a maximum likelihood estimate) from long-term exposure to BD was 9×10^{-3} per ppm. This means that for every 1,000 people exposed continuously for a lifetime to 1 ppm BD, one would expect 9 deaths from cancer caused by BD exposure in addition to the number of deaths expected from other cancer causes. The impact of data and modeling assumptions on the cancer risk estimates is illustrated by comparing the estimate with the Agency's previous risk estimate of 2.5×10^{-1} per ppm (or 9 deaths in 1,000 compared with 250 deaths in 1,000), which was the upper bound estimate obtained using tumor data from mice (the more sensitive rodent species) (U.S. EPA 1985).

Quantitative risk assessments have very important implications for regulatory policies. In the case of BD, the unit cancer risk of 2.5×10^{-1} per ppm was used by the EPA's Office of Mobile Sources in its *Motor Vehicle Air Toxics Study*. This study (which was released in 1993 and will be updated in 2000) evaluated the overall cancer risks posed by exposure to mobile-source emissions, including the separate risks from exposure to BD, benzene, formaldehyde, acetaldehyde, and diesel exhaust (U.S. EPA 1993). The 1993 document indicated that BD presented the highest risk of the substances evaluated. If the cancer risk factor for BD is lower, as was suggested in the Agency's 1998 draft health risk assessment, the relative ranking of BD among the mobile-source air toxics would change. This demonstrates the dependence of cancer risk estimates on the data set chosen for the exposure-response analyses and the difficulty in developing reliable risk estimates in the absence of key information. To reduce these uncertainties, the EPA's new draft guidelines for cancer risk assessment (U.S. EPA 1996) encourage using information about biological mechanisms when available and better measures of exposure (such as biomarkers, biologic indicators of past or current exposure to chemicals) to estimate the risks of exposures to carcinogens.

HEI'S BUTADIENE RESEARCH PROGRAM

HEI's air toxics research program includes research on butadiene as well as benzene, aldehydes, diesel exhaust, and polycyclic organic matter. The Institute first identified research needs for air toxics from mobile sources at a 1992 workshop organized by HEI with broad participation from individuals in industry, regulatory agencies, and academia (HEI 1993b). The EPA and members of the motor vehicle industry had urged HEI to conduct that workshop and to prepare a report to (1) complement the Agency's study (U.S. EPA 1993) required under Section 202(l) of the 1990 Amendments to the Clean Air Act, and (2) help focus research on certain unregulated mobile-source emissions. The goal of the workshop and the research recommendations presented in the HEI Communication (HEI 1993b) was to provide decision makers with suggestions for research that could narrow the uncertainties related to assessing health risks from specific air toxics. Workshop participants discussed research needs for aldehydes, benzene, BD, methanol, and polycyclic organic matter. These chemicals were chosen on the basis of their listing in the Clean Air Act and their potentials for adverse health consequences.

On the basis of information gathered at the workshop, HEI issued Request for Applications (RFA) 93-1, *Novel Approaches to Extrapolation of Health Effects for Mobile Source Toxic Air Pollutants* (HEI 1993a). This RFA identified high-dose to low-dose and cross-species extrapolation as research issues of interest. Within the context of the issues identified at the workshop and given the applications in response to the RFA, HEI established a research program for BD that included developing methods for estimating internal dose, comparing animal and human metabolism, and identifying biomarkers of exposure or effect in animals that could eventually be used in human studies. (Details on HEI's research programs in these areas can be found in HEI Program Summaries for Benzene and 1,3-Butadiene [HEI 1995] and for Air Toxics [HEI 1999]).

The principal objective of HEI's initial BD research program was to obtain data or develop methods that would improve the ability to extrapolate the health effects found in animals or humans exposed to high levels of toxic pollutants to those expected in humans after exposure to low ambient levels. To accomplish this, it would be necessary to elucidate the roles of different metabolites in BD-induced carcinogenesis and to develop methods for improving exposure and risk assessment by:

- evaluating the mutagenic potency and carcinogenicity of BD metabolites in rats and mice;
- identifying and characterizing the differences between mice and rats in their formation of DNA adducts induced by BD metabolites; and
- identifying and characterizing biomarkers of exposures and effects.

Four of the investigators (Henderson, Recio, Walker, and Blair) whose studies are reported here were among the applicants who submitted proposals in response to RFA 93-1. These proposals, which underwent peer review by an expert panel and the HEI Health Research Committee, were selected for funding because of their technical merit and because of their potential to address the Institute's research goals with an integrated approach. Henderson and colleagues exposed mice and rats to BDO_2 by inhalation to determine the carcinogenic potency of this metabolite in the two species. The two investigator teams led by Recio and Walker examined the in vivo mutagenicity and mutational spectra (patterns of changes in DNA) caused by BD, BDO, and BDO_2 in mice and rats. Recio's team also examined the mutational spectra induced by BDO and BDO_2 in vitro using human and rat cells. By comparing BD-induced events at the molecular level, these investigators hoped to identify which of the reactive metabolites are responsible for BD-induced carcinogenicity and for the interspecies differences in sensitivity to BD. Blair and associates developed methods for measuring certain DNA adducts derived from BD and its metabolites in mice and rats with the goals of understanding differences between species in their reactivity to BD metabolites and of identifying possible biomarkers of BD exposures and effects. The fifth study by Swenberg and colleagues (which was funded under HEI's Preliminary Application process RFPA 95-2) analyzed certain adducts formed between BD metabolites and the blood protein hemoglobin and ascertained whether these adducts could be detected in humans who had been exposed to BD.

Before the research started, HEI convened a workshop in which four of the five principal investigators met to discuss their individual studies and possible collaborations. Although HEI's butadiene program was conceived and designed as a group of individual yet complementary studies, the investigators modified their study designs to facilitate comparing their results. HEI fostered continuing interactions among investigators by convening annual workshops and meetings held at the HEI Annual Conferences. These interactions led to collaborations among the investigators that are evidenced by joint publications (Meng et al. 1996, 1997, 1998a,b, 1999a,b; Tretyakova et al. 1997, 1998a,b; Koc et al. 1999; Oe et al. 1999). Collaborations among the investigators resulted in a single route of animal exposure (inhalation), the same exposure levels,

Table 1. Summary of Animal Inhalation Exposure Conditions, Study Endpoints, and Investigators

Exposure Parameters[a]					Endpoints by Investigator			
						Mutations in Selected Tissues	Adducts	
Chemical	Laboratory[b]	Concentration (ppm)	Duration (weeks)	Species	Tumors		DNA	Hemoglobin
BD	Haskell Laboratory	0; 1,000	13	Crl:CD®BR rats $B6C3F_1$ mice				Swenberg
BD	CIIT (Recio)	0; 1,250	1 day to 2 weeks	F344 rats $B6C3F_1$ mice		Walker	Blair	Swenberg
BD	LRRI (Henderson)	0; 20; 62.5; 625	2, 4	F344 rats $B6C3F_1$ mice		Walker		
BDO	LRRI (Henderson)	0; 2.5; 25	4	F344 rats $B6C3F_1$ mice		Recio[c], Walker		
BDO_2	LRRI (Henderson)	0; 2.5; 5	4	Sprague-Dawley rats; $B6C3F_1$ mice	Henderson			
BDO_2	LRRI (Henderson)	0; 2; 4	4	F344 rats $B6C3F_1$ mice		Recio[c], Walker		

[a] All exposures were by inhalation, 6 hours/day, 5 days/week for the duration indicated.

[b] Haskell Laboratory was not part of HEI's butadiene program. CIIT = Chemical Industry Institute of Toxicology; LRRI = Lovelace Respiratory Research Institute. Exposures were conducted under the guidance of the investigators named.

[c] Recio used transgenic animals (rodents into which a bacterial target gene has been introduced for ease of cellular analysis).

and when possible, common animal exposures, thus providing an opportunity to make comparisons across studies (Table 1). The animals were exposed at either the Chemical Industry Institute of Toxicology (1,250 ppm BD) or the Lovelace Respiratory Research Institute (all other BD exposure levels and the BDO and BDO_2 exposures).

When the Research Committee funded this set of studies, it also recognized that studies in human populations would be needed to validate some of the more promising biomarkers being developed. Therefore, HEI extended its butadiene program to support a study in the Czech Republic led by Dr. Richard Albertini to test the feasibility of using various proposed biomarkers, including several developed in this research program. The aim of this effort (completed in 1999) was to determine if it is possible to identify a dose-response relation between the level of an individual's exposure and the amount of a biomarker detected in blood or urine. If the feasibility study shows a high degree of correlation between the level of BD exposure and the concentration of any biomarker, a full epidemiologic study would need to be done to ascertain if the biomarker is useful for predicting health outcomes.

This HEI Research Report contains the Investigators' Reports for the five BD-related studies funded under RFA 93-1 and RFPA 95-2. As shown in Figure 3, these five studies were designed to fill gaps in the exposure-dose-response paradigm and thus to improve the scientific basis for BD risk assessment.

The investigators submitted draft final reports in 1998 for review by the Institute's Health Review Committee. The draft reports underwent external peer review by experts in toxicology, carcinogenesis, and metabolism, and were discussed by the HEI Review Committee. The purpose of this review was to ensure the quality of the investigators' research and to interpret the relevance of the work in the context of policy and regulatory issues. On the basis of the comments from external reviewers and its own discussions, the Review Committee requested revised reports, which the investigators submitted later that year. The Committee accepted the revised reports for publication in the HEI Research Report series. The Review Committee and staff also prepared three Commentaries: one about the carcinogenicity study (Henderson), one about the mutagenicity studies (Recio and Walker), and one about the studies of development of biomarkers (Blair and Swenberg). These Commentaries are intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the studies and by placing the investigators' findings into scientific and regulatory perspective.

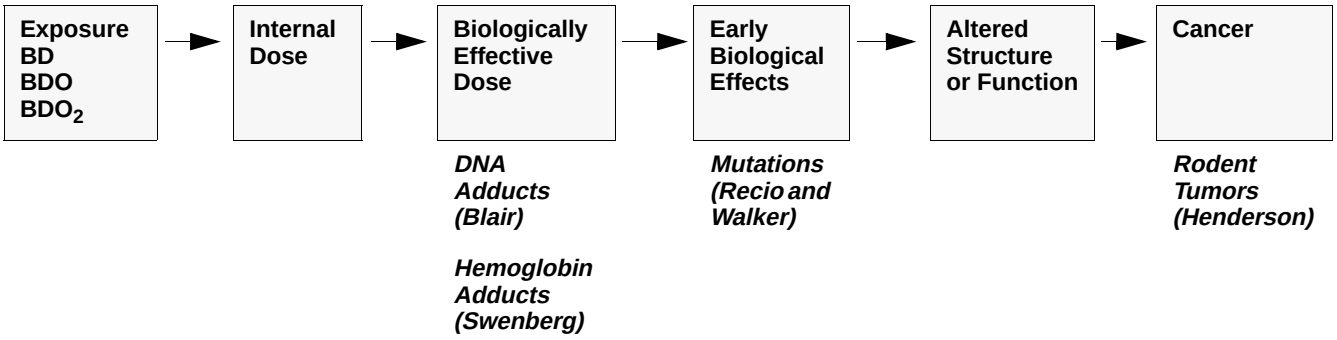

Figure 3. The exposure-dose-response paradigm and where the five HEI butadiene research projects fit within its context.

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REFERENCES

Delzell E, Sathiakumar N, Hovinga M, Macaluso M, Julian J, Larson R, Cole P, Muir DCF. 1996. A follow-up study of synthetic rubber workers. Toxicology 113:182–189.

Delzell E, Sathiakumar N, Macaluso M. 1995. A Follow-Up Study of Synthetic Rubber Workers. International Institute of Synthetic Rubber Producers, Houston, TX.

Divine BJ. 1990. An update on mortality among workers at a 1,3-butadiene facility: Preliminary results. Environ Health Perspect 86:119–128.

Divine BJ, Hartman CM. 1996. Mortality update of butadiene production workers. Toxicology 113:169–181.

Divine BJ, Wendt JK, Hartman CM. 1993. Cancer mortality among workers at a butadiene production facility. In: Butadiene and Styrene: Assessment of Health Hazards, IARC Scientific Publication Number 127 (Sorsa M, Peltonen K, Vainio H, Hemminiki K, eds.) pp. 345–362. International Agency for Research on Cancer, Lyon, France.

European Centre for Ecotoxicity and Toxicology of Chemicals. 1997. 1,3-Butadiene OEL Criteria Document, 2nd ed. European Centre for Ecotoxicity and Toxicology of Chemicals, Brussels, Belgium.

Health Effects Institute. 1993a. Novel Approaches to Extrapolation of Health Effects for Mobile Source Toxic Air Pollutants. Request for Applications 93-1. Health Effects Institute, Cambridge, MA.

Health Effects Institute. 1993b. Research Priorities for Mobile Source Air Toxics. HEI Communication Number 2. Health Effects Institute, Cambridge, MA.

Health Effects Institute. 1995. Research on Benzene and 1,3-Butadiene. HEI Program Summary. Health Effects Institute, Cambridge, MA.

Health Effects Institute. 1999. Research on Air Toxics. HEI Program Summary. Health Effects Institute, Cambridge, MA

Himmelstein MW, Acquavella JF, Recio L, Medinsky MA, Bond JA. 1997. Toxicology of 1,3-butadiene. Crit Rev Toxicol 27:1–108.

International Agency for Research on Cancer. 1992. Occupational Exposures to Mists and Vapours from Strong Inorganic Acids and Other Industrial Chemicals. IARC Monogr Eval Carcinog Risk Chem Hum 54:237–285.

International Agency for Research on Cancer. 1999. Re-evaluation of Some Organic Chemicals, Hydrazine and Hydrogen Peroxide, Part I. IARC Monogr Eval Carcinog Risk Chem Hum 71:109–125.

International Institute of Synthetic Rubber Producers. 1981. The Toxicity and Carcinogenicity of Butadiene Gas Administered to Rats by Inhalation for Approximately 24 Months. Report No. 2653-5222/2. International Institute of Synthetic Rubber Producers, Houston, TX.

Koc H, Tretyakova NY, Walker VE, Swenberg JA. 1999. Molecular dosimetry of N-7-guanine adduct formation in mice and rats exposed to 1,3-butadiene. Chem Res Toxicol 12:566–574.

Koivisto P, Kilpeläinen I, Rasanen I, Adler I-D, Pacchierotti F, Peltonen K. 1999. Butadiene diolepoxide- and diepoxybutane-derived DNA adducts at N7-guanine: A high occurrence of diolepoxide-derived adducts in mouse lung after 1,3-butadiene exposure. Carcinogenesis 20:1253–1259.

Krause R, Elfarra AA. 1997. Oxidation of butadiene monoxide to *meso*- and (±)-diepoxybutane by cDNA-expressed human cytochrome P450s and by mouse, rat, and human liver microsomes: Evidence for preferential hydration of *meso*-diepoxybutane in rat and liver microsomes. Arch Biochem Biophys 337:176–184.

Macaluso M, Larson R, Delzell E, Sathiakumar N, Hovinga M, Julian J, Muir D, Cole P. 1996. Leukemia and cumulative exposure to butadiene, styrene and benzene among workers in the synthetic rubber industry. Toxicology 113:190–202.

Matanoski GM, Santos-Burgoa C, Schwartz L. 1990. Mortality of a cohort of workers in the styrene-butadiene polymer manufacturing industry. Environ Health Perspect 86:107–117.

Matanoski GM, Schwartz L. 1987. Mortality of workers in styrene-butadiene polymer production. J Occup Med 29:675–680.

Meinhardt TJ, Lemen RA, Crandall MS, Young RJ. 1982. Environmental epidemiologic investigation of the styrene-butadiene rubber industry: Mortality patterns with discussion of the lymphohematopoietic and lymphatic malignancies. Scand J Work Environ Health 8:250–259.

Melnick RL, Kohn MC. 1995. Mechanistic data indicate that 1,3-butadiene is a human carcinogen. Carcinogenesis 16:157–163.

Meng Q, Henderson RH, Reilly AA, Bauer M, Chen T, Heflich RH, Walker VE. 1999a. Mutagenicity of 1,3-butadiene at the *hprt* locus of T-cells following inhalation exposures of female mice and rats. Mutat Res 429:107–125.

Meng Q, Henderson RF, Reilly AA, Bauer M, Walker VE. 1998a. Relative contribution of epoxybutene versus diepoxybutane to the mutagenicity of butadiene following inhalation exposures of mice and rats (abstract). Toxicologist (Suppl 1) 42:182.

Meng Q, Henderson RF, Reilly AA, Bauer M, Walker VE. 1999b. Mutagenicity of epoxybutene and diepoxybutane at the *hprt* locus of T-cells following inhalation exposures of mice and rats. Mutat Res 429:127–140.

Meng Q, Henderson RF, Walker VE. 1997. Mutagenicity at *hprt* in splenic T-cells of mice and rats exposed by inhalation to diepoxybutane (abstract). Toxicologist (Suppl 1) 36:308.

Meng Q, Recio L, Reilly AA, Wong BA, Bauer M, Walker VE. 1998b. Comparison of the mutagenic potency of 1,3-butadiene at the *hprt* locus of T-lymphocytes following inhalation exposure of female B6C3F_1 mice and F344 rats. Carcinogenesis 19:1019–1027.

Meng Q, Recio L, Wong B, Walker VE. 1996. Comparison of the mutagenic potency of butadiene at *hprt* of T-cells following inhalation exposures of B6C3F_1 mice and F344 rats (abstract). Environ Mol Mutagen (Suppl) 27:48.

National Toxicology Program. 1984. Toxicology and Carcinogenesis Studies of 1,3-Butadiene (CAS No. 106-99-0) in B6C3F_1 Mice (Inhalation Studies). NTP TR 288, NIH Pub. No. 84-2544. U.S. Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC.

National Toxicology Program. 1992. Toxicology and Carcinogenesis Studies of 1,3-Butadiene (CAS No. 106-99-0) in B6C3F_1 Mice (Inhalation Studies). NTP TR 434, NIH Pub. No. 93-3165. U.S. Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC.

National Toxicology Program. 1998a. Eighth Report on Carcinogens: 1998 Summary. U.S. Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC.

National Toxicology Program. 1998b. Toxicology and carcinogenesis studies of 1,3-butadiene (CAS No. 106-99-0) in B6C3F_1 mice (inhalation studies). Fed Regist 63:13418.

Nieusma JL, Claffey DJ, Maniglier-Poulet C, Imiolczyk T, Ross D, Ruth JA. 1997. Stereochemical aspects of 1,3-butadiene metabolism and toxicity in rat and mouse liver microsomes and freshly isolated rat hepatocytes. Chem Res Toxicol 10:450–456.

Nieusma JL, Claffey DJ, Ruth JA, Ross D. 1998. Stereochemical aspects of the conjugation epoxide metabolites of butadiene with glutathione in rat liver cytosol and freshly isolated rat hepatocytes. Toxicol Sci 43:102–109.

Occupational Safety and Health Administration. 1990. Occupational Exposure to 1,3-Butadiene. Fed Regist 55:32737–32757.

Oe T, Kambouris SJ, Walker VE, Meng Q, Recio L, Wherli S, Chaudhary AK, Blair IA. 1999. Persistence of N7-(2,3,4-trihydroxybutyl)guanine adducts in the livers of mice and rats exposed to 1,3-butadiene. Chem Res Toxicol 12:247–257.

Richardson KA, Peters MM, Wong BA, Megens RH, van Elburg PA, Booth ED, Boogaard PJ, Bond JA, Medinsky MA, Watson WP, van Sittert NJ. 1999. Quantitative and qualitative differences in the metabolism of ^{14}C-1,3-butadiene in rats and mice: Relevance to cancer susceptibility. Toxicol Sci 49:186–201.

Sathiakumar N, Delzell E, Hovinga M, Macaluso, Julian JA, Larson R, Cole P, Muir DCF. 1998. Mortality from cancer and other causes of death among synthetic rubber workers. Occup Environ Med 55:230–235.

Tretyakova NY, Chang SU, Walker V, Hass JR, Swenberg JA. 1997. DNA adducts in TK6 cells and tissues of rats and mice exposed to 1,3-butadiene and its metabolites (abstract). Proc Am Assoc Cancer Res 38:333.

Tretyakova NY, Chiang SU, Walker VE, Swenberg JA. 1998a. Quantitative analysis of 1,3-butadiene-induced DNA adducts in vivo and in vitro using LC–EST + MS/MS. J Mass Spectrom 33:363–376.

Tretyakova NY, Walker VE, Swenberg JA. 1998b. Formation and persistence of DNA adducts in liver of rats and mice exposed to 1,3-butadiene (abstract). Toxicologist (Suppl 1) 42:180.

U.S. Environmental Protection Agency. 1985. Mutagenicity and Carcinogenicity Assessment of 1,3-Butadiene. EPA/600/8-85/004F. Office of Research and Development, Washington, DC.

U.S. Environmental Protection Agency. 1993. Motor Vehicle–Related Air Toxics Study. EPA/420-R-93-005. Office of Mobile Sources, Ann Arbor, MI.

U.S. Environmental Protection Agency. 1996. Draft Guidelines for Carcinogen Risk Assessment. Fed Regist 61:17960–18011.

U.S. Environmental Protection Agency. 1998a. An SAB Report: Review of the Health Risk Assessment of 1,3-Buta-

diene. EPA-SAB-EHC-99-003. Science Advisory Board, Washington, DC.

U.S. Environmental Protection Agency. 1998b. Health Risk Assessment of 1,3-Butadiene (External Review Draft). Office of Research and Development, Washington, DC.

U.S. Environmental Protection Agency. 1999. Integrated Urban Air Toxics Strategy. Fed Regist 64(137):38705–38740.

1,3-Butadiene: Cancer, Mutations, and Adducts

Part I: Carcinogenicity of 1,2,3,4-Diepoxybutane

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ABSTRACT

Reports in the literature suggest that one reason for the greater sensitivity of mice to the carcinogenicity of 1,3-butadiene (BD)* is that exposed mice metabolize much more of the BD to 1,2,3,4-diepoxybutane (BDO_2) than do exposed rats. The purpose of this study was to determine the tumorigenicity of BDO_2 in rats and in mice exposed to the same concentration of the agent. Female $B6C3F_1$ mice and Sprague-Dawley rats, 10 to 11 weeks old, 56 per group, were exposed by inhalation to 0, 2.5, or 5.0 ppm BDO_2, 6 hours/day, 5 days/week for 6 weeks. Preliminary dosimetry studies in rodents exposed for 6 hours to 12 ppm BDO_2 indicated that blood levels would be expected to be approximately 100 and 200 pmol/g at the two exposure concentrations in the rat and twice those levels in the mouse. During the 6-week exposure, the mice at the high exposure level showed signs of labored breathing during the last week, and four mice died. In the others, however, the respiratory symptoms disappeared after exposure ended. Rats showed no clinical signs of toxicity during exposure but developed labored breathing after the end of the exposure leading to the death of 13 rats within 3 months.

At the end of the exposure, some animals (8 per group) were evaluated for the acute toxicity resulting from the BDO_2 exposure. The remaining exposed rats and mice were held for 18 months for observation of tumor development. At the end of the exposure, rats had no biologically significant alteration in standard hematological parameters, but mice had a dose-dependent increase in neutrophils and decrease in lymphocytes. In both species the significant histopathologic lesions were in the nose, concentrated around the main airflow pathway. Necrosis, inflammation, and squamous metaplasia of the nasal mucosa, as well as atrophy of the turbinates, were all present at the end of exposure to 5.0 ppm. Within 6 months, necrosis and inflammation subsided, but squamous metaplasia remained in the mice. In rats that died after exposure, squamous metaplasia was seen in areas of earlier inflammation and, in other rats, extended beyond those areas with time. The metaplasia was severe enough to restrict and occlude the nasopharyngeal duct. Later, keratinizing squamous cell carcinomas developed from the metaplastic foci in rats but not mice. At the end of 18 months, the only significant increase in neoplasia in the exposed rats was a dose-dependent increase in neoplasms of the nasal mucosa (0/47, 12/48, and 21/48 for the control, 2.5 ppm, and 5.0 ppm exposures, respectively). Neoplasia of the nasal mucosa did not increase significantly in the mice; neoplastic lesions in the mice were observed in reproductive organs, lymph nodes, bone, liver, Harderian gland, pancreas, and lung. The only significant increase in neoplasms in a single organ in the mice was in the Harderian gland (0/40, 2/42, and 5/36 for the control, 2.5 ppm, and 5.0 ppm exposures, respectively). This tumor accounts for the apparent trend toward an increase in total neoplastic lesions in mice as a function of dose (10/40, 7/42, and 16/36 for control, 2.5 ppm, and 5.0 ppm exposures, respectively).

These findings indicate that the metabolite of BD, BDO_2, is carcinogenic in the respiratory tract of rats. An increase in respiratory tract tumors was not observed in similarly exposed mice despite the fact that preliminary studies indicated mice should have received twice the dose to tissue compared with the rats. High cytosolic activity of detoxication enzymes in the mouse may account, in part, for the differences in response.

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

This Investigators' Report is Part I of Health Effects Institute Research Report Number 92, which also includes a Commentary by the Health Review Committee about Part I, four additional Investigators' Reports (Parts II through V) by different research groups, a Commentary on Parts II and III, a Commentary on Parts IV and V, and an HEI Statement about the five butadiene research projects reported here. Correspondence regarding the Part I Investigators' Report may be addressed to Dr. Rogene Henderson, Lovelace Respiratory Research Institute, P.O. Box 5890, Albuquerque, NM 87185.

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INTRODUCTION

Butadiene, a widely used industrial chemical, has been reported to be carcinogenic in animal bioassays. However, there is a large species difference in the response of $B6C3F_1$ mice and Sprague-Dawley rats to inhaled BD (Huff et al. 1985; Owen et al. 1987; Melnick et al. 1990). Recent evidence indicates that this may be due to differences in the metabolism of BD between these two species. 1,3-Butadiene is biotransformed into a reactive intermediate, BDO_2, a suspected potent carcinogen. The observation that sensitive animals produce more BDO_2 than insensitive animals suggests that BDO_2, rather than BD, may be the primary carcinogen and should be of more concern. Since risk assessment of BD in humans is based on animal data, an investigation of BDO_2 carcinogenicity and possible differences among species is warranted.

Mice appear to be more sensitive to the tumorigenic effects of inhaled BD than rats. In chronic exposure studies, $B6C3F_1$ mice exposed to high levels of BD (200 ppm or higher) had increased lethal lymphocytic lymphomas (Huff et al. 1985). At lower exposure concentrations, the mice had increased lung neoplasia (at 6.25 ppm BD in females and 62.5 ppm BD in males) and hemangiosarcomas of the heart (62.5 ppm BD in males) (Melnick et al. 1990). Increased neoplasias in the liver, mammary gland, and ovary were also observed. Besides neoplasia, the exposed mice had macrocytic anemia. When chronically exposed to BD over a 2-year period, Sprague-Dawley rats had increased tumors in mammary glands at the 1,000 ppm dose level and increased neoplasia in the thyroid, testis, uterus, pancreas, Zymbal's gland, and mammary glands after exposures to 8,000 ppm (Owen and Glaister 1990). The exposed rats did not develop macrocytic anemia or lymphomas. Thus, responses of the two species differed in both the level of BD required to induce neoplasia and the site of tumor induction.

A potential reason for the striking species differences in response to BD exposure is differences in the metabolism of BD. There is ample evidence for quantitative differences in the metabolism of BD among various species. The in vitro work of Schmidt and Loeser (1985), later confirmed by Csanády and associates (1992), showed that mouse-liver microsomes have a greater capacity than do liver microsomes of rats or humans to form the initial metabolite of BD, 1,2-epoxy-3-butene (BDO), and a lesser capacity for hydrolysis of BDO. Studies conducted in vivo indicated lower levels of BDO in the blood of BD-exposed rats and monkeys than in mice (Bond et al. 1986; Dahl et al. 1991). However, the species differences in the formation and removal of BDO do not appear to be sufficient to explain such large differences in the response to BD.

Recent evidence suggests much larger species differences in the formation of BDO_2. Csanády and coworkers (1992) found that only mouse livers, but not rat or human livers, could oxidize BDO to BDO_2. In studies at the Lovelace Respiratory Research Institute (LRRI) and at the Chemical Industry Institute of Toxicology, BDO_2 could be detected in the blood of mice but not of rats exposed to low levels of BD (100 ppm or less for 4 to 6 hours) (Bechtold et al. 1995; Himmelstein et al. 1994, 1995). However, in more recent studies at LRRI (Thornton-Manning et al. 1995a,b), using a more sensitive method, a small amount of BDO_2 was found in BD-exposed rats. Vangala and coworkers (1993) observed DNA cross-linking, an effect that could be caused by BDO_2 but not by BDO, in the livers of BD-exposed mice but not in rats. These data suggest that BDO_2 may be the potent genotoxic metabolite of BD. This could provide at least one explanation for the large species differences in response to inhaled BD.

Studies by Boogaard and Bond (1996) and Boogaard and coworkers (1996) indicate there are also species differences in the detoxication routes for the BDO_2. Human liver microsomes hydrolyze the diepoxide faster than do rat liver microsomes, which in turn are faster than mouse liver microsomes (Boogaard and Bond 1996). The detoxication via cytosolic conjugation with glutathione follows the reverse order, with mouse liver cytosol catalyzing the reaction faster than does the rat liver cytosol, which is faster than the human liver cytosol catalysis.

Previous studies have shown that lung tumors induced by BD contain an activated K-*ras* gene (Goodrow et al. 1990). This gene activation was the result of a codon 13 mutation (a G → C transversion in the first base) within exon 1. The authors suggested that this specific mutation may represent a target for BD mutagenesis in mice since such mutations are not found in spontaneously occurring lung tumors, liver tumors, or lymphomas, or in tumors induced by radiation or chemical carcinogens such as methylnitrosourea, benzo[*a*]pyrene, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone, ethylnitrosourea, dimethylnitrosamine, and vinyl chloride.

The prevalence of activation of the K-*ras* gene has not been examined in the tumors from rats exposed to BD. However, K-*ras* mutations have been reported at varying frequencies in lung tumors induced by inhaled plutonium, x-irradiation, azomethane, nitrosomethylurea, and tetranitromethane in rats. Thus, that pathway to carcinogenesis is operative in rats.

The current study was designed to determine the responses of rats and of mice following direct exposure to BDO_2.

SPECIFIC AIMS

This study tests the hypothesis that BDO_2 is as tumorigenic in rats as in mice when both species are directly exposed to the metabolite.

This study has two specific aims:

1. To determine the carcinogenicity of BDO_2 repeatedly administered by inhalation to (a) the susceptible rodent species, the $B6C3F_1$ mouse, which is known to generate BDO_2 from inhaled BD; and (b) the resistant rodent species, the Sprague-Dawley rat, which generates very little BDO_2 from inhaled BD.
2. To assay for the K-*ras* codon 13 mutation as a marker of BD-specific mutations and determine if BDO_2 induces DNA adducts leading to the K-*ras* mutations already demonstrated in lung tumors from BD-exposed mice.

METHODS AND STUDY DESIGN

GENERAL DESIGN

$B6C3F_1$ mice and Sprague-Dawley rats were used because large differences in response to BD have been observed between these species in BD chronic inhalation bioassays. For economic reasons, we chose to use only one sex. The female was chosen because the female was the more sensitive sex for induction of lung tumors in the 2-year bioassay studies (Huff et al. 1985; Owen et al. 1987; Melnick et al. 1990).

Preliminary dosimetry studies funded by the Chemical Manufacturers Association (see Appendix A for a complete report) allowed us to estimate the dose to lung and blood when BDO_2 was administered intraperitoneally (i.p.), intratracheally (i.t.), or by inhalation. The half-life of BDO_2 in saline at 37°C was also measured to determine if delivery of BDO_2 via osmotic pumps would be feasible.

These preliminary studies provided the following information:

1. BDO_2 cleared equally rapidly from the rat and mouse lungs with a half-life of less than 5 minutes.
2. BDO_2 instilled in the lung reached the blood for distribution to other organs, indicating that inhaled BDO_2 may induce tumors in organs other than the lung.
3. As had been expected, higher blood and lung levels of BDO_2 were achieved by i.t. instillation rather than by i.p. injection.
4. Inhalation exposures achieved higher doses of BDO_2 in the blood and lungs of mice than in rats (approximately twofold higher in mice). This would be expected on the basis of breathing rates per kilogram of body weight in the two species.
5. Inhalation exposure to 12 ppm BDO_2 for 6 hours resulted in levels of BDO_2 in the blood of mice (at the end of exposure) equivalent to those seen 30 minutes after i.t. instillation of 26 mg/kg BDO_2.
6. The half-life of the BDO_2 in saline at 37°C was only 60 hours. Thus, we would have to replace osmotic pumps surgically too often to make their use practical.
7. A single 6-hour inhalation exposure of rats and mice to 18 ppm BDO_2 produced minimal lesions in the lungs, but there was focal necrosis in the nose at the anterior dorsal epithelium. The lesions had begun to repair 7 days after the exposure. No lesions were found in liver, kidney, bone marrow, heart, or ovary.

We chose inhalation as the route of exposure for several reasons. First, we hypothesized that the BDO_2 would be a direct-acting carcinogen that is most likely to cause tumors near the site of administration. Second, the lung and heart were the two most sensitive sites for induction of tumors in the mice exposed to BD, and we were especially interested in delivering sufficient doses to those sites. Third, we wished to avoid the repeated bolus dosing involved in i.t. depositions and preferred the slower dose rate provided by inhalation.

A study was designed and initiated in which rats and mice were to be exposed to 0 (control), 10, or 20 ppm BDO_2, 6 hours/day, 5 days/week for 4 weeks. These concentrations of BDO_2 proved to be too toxic for repeated exposures. As might be expected, the nasal tissue was a major target site because the BDO_2 is a water-soluble, highly reactive compound. Rats and mice are obligate nose breathers and cannot tolerate occlusion of the nasal air passage; death occurred as a result of occlusion of the upper respiratory tract. Therefore, the study was redesigned with the exposure levels reduced to 0, 2.5, and 5.0 ppm and the exposure duration extended to 6 weeks.

Design of Study

Animals (mice and rats) were approximately 6 weeks old when the exposures began. They were 4 weeks old at the time of receipt and were quarantined for 2 weeks after receipt and prior to exposures. Animal usage in this study is presented in Table 1. A total of 168 Charles River $B6C3F_1$ female mice and 168 Charles River Sprague-Dawley female rats were used. Groups of 56 animals were exposed to 0, 2.5, and 5.0 ppm BDO_2. Animals were exposed 6 hours/day, 5 days/week for 6 weeks. Following exposure, the animals were held for a time to be deter-

Table 1. Planned Distribution of Rats and Mice Among Treatment Groups

	BDO_2 Exposure Group[b] (ppm)		
Observation Period[a]	0	2.5	5.0
6 Weeks	8	8	8
6 Months	4	4	4
12 Months	4	4	4
18 months	40	40	40

[a] "6 Weeks" was at the end of the exposure period. All durations in months indicate time after the exposure ended. All animals were to be killed at the end of the observation period.

[b] $n = 56$ for each exposure concentration.

mined by scheduled deaths at 6 and 12 months. From each exposure group, 16 animals were scheduled to be killed to determine (1) the subacute toxicity of BDO_2 at the end of exposure (8 animals/group), and (2) the length of the observation period required to observe tumors (4 animals/group at 6 and 12 months after exposure). Due to unexpected mortality, not all of the interim-death schedules were met (see section on Deaths and Necropsies). The end-of-study scheduled deaths of the animals occurred 18 months after the end of the exposures. Four animals were used for serological testing before the study started.

Animal Husbandry

As stated above, the species, strains, and sex of the animals used in the study were selected because of their sensitivities to BD inhalation in 2-year rodent bioassays. Inhalation was chosen because it is the best route of exposure to get a reasonably slow delivery (to avoid acute toxicity) of the BDO_2 to the lung, the most sensitive organ in the bioassays. Delivery via the lung also avoids the first-pass metabolism of the compound in the liver.

Animals were randomly assigned to each exposure group (including the air control group) by species and by weight within 6 days before the start of exposures. Randomization was accomplished using the Path/Tox Computer System (Xybion Corporation, Cedar Knolls, NJ). Rats and mice were individually identified by tail tattoo. All treatment procedures had been approved by our Animal Care Committee.

Mice and rats were individually housed in stainless-steel, wire-mesh cages within Hazleton-2000 chambers (H2000, Lab Products, Inc., Aberdeen, MD) during quarantine and exposures. Cage racks were rotated clockwise during the weekly transfer of animals to clean chambers.

Each cage unit had an excreta tray lined with cageboard treated to reduce bacterial growth and the formation of ammonia from urine. The cageboard was changed twice daily (before and after the exposure) and the excreta trays washed daily to keep ammonia concentrations within the chamber as low as possible. Once every week animals were transferred to clean racks within clean chambers.

The light/dark cycle was 12 hours light/12 hours dark with lights on at 0600. Standard fluorescent lights were used. Temperature and relative humidity were monitored every 5 minutes by computer. The chamber temperature was maintained at 75 ± 3°F and the relative humidity at 40% to 70%. At the end of the 6-week exposure, animals were housed in polycarbonate cages (3 mice/cage and 2 rats/cage) containing hardwood-chip bedding and filter caps. Food (Lab-Blox, Allied Mills, Chicago, IL) and water from bottles with sipper tubes were provided ad libitum.

The health of the animals was assured during the last few days of the quarantine. Four animals/species were killed 1 to 3 days before the animals were to be released from quarantine. All the necropsied animals were examined grossly for disease and parasites. Sera from these animals were tested for antibodies to pneumonia virus, Sendai virus, RCV/SDA, reovirus type 3, KRV, H-1, choriomeningitis, *M. pulmonis*, CAR Bacillus, and orphan parvovirus (Microbiological Associates, Inc., Rockville, MD).

The rats and mice were fed Teklad Certified Rodent Diet (Harlan Teklad, Madison, WI). During the first week of quarantine, food was provided ad libitum. During the second week of quarantine, and until exposures began, food trays were removed at the time of the morning chamber check and replaced during the afternoon check. Once exposures began, food was withheld during exposure hours. Water was supplied ad libitum from an automatic watering system, which was checked daily for proper function. When the animals were housed in polycarbonate shoe-box cages, food and water were available ad libitum.

BDO_2 INHALATION EXPOSURE SYSTEM

Test Compound

BDO_2 (CAS #30031-64-2) was obtained from Aldrich Chemical Co. (Milwaukee, WI). The compound was a racemic mixture of the stereoisomers of BDO_2 with none of the *meso* form present. BDO_2 is a liquid with a molecular weight of 86.1 and density of 1.113. Its LD_{50} in mice is 61 mg/kg (i.p.), and its LC_{50} in rats is 90 ppm (4 hours). A minimum purity of 97% BDO_2 was required for the exposure. Purity of the compound was checked before the exposure began; the commercial product (> 97% pure)

was further purified to > 99% by redistillation. Purity was determined by nuclear magnetic resonance analysis.

Chamber Conditions

Exposures were conducted in stainless-steel Hazelton-2000 inhalation chambers (H2000, Lab Products, Inc., Aberdeen, MD). The rate of airflow through these chambers was 15 ± 2 ft^3/min, which provided 15 chamber-air changes/hour. All chamber supply air was HEPA-filtered before being introduced into the chamber supply system.

Vapor Generation

BDO_2 vapors were generated using a heated J tube (140°C) located within a Lexan enclosure connected to the facility exhaust. The liquid BDO_2 was supplied to a heated J tube using a syringe pump at a controlled feed rate. Nitrogen (N_2) was used to carry the vapors from the tube to the chamber. The temperature of the J tube, the BDO_2 feed rate to the J tube, and the flow rate of the carrier gas were optimized to provide for stable vapor concentrations within the chambers throughout the 6-hour daily exposure period. Chambers were maintained at negative pressure relative to the room to protect personnel, who wore respirators while the exposures were in progress. Exhaust from the chambers was cleaned by passage through a charcoal filter followed by a backup charcoal filter.

Monitoring of BDO_2

BDO_2 vapor concentration in each exposure chamber was monitored periodically by infrared (IR) absorbance using a MIRAN-IA IR spectrometer (The Foxboro Company, Foxboro, MA) to ensure that the relative concentration was stable throughout the exposure day. Vapor concentration was determined by extracting chamber air through a bubbler system that consisted of two 100-mL–volume glass impingers connected in series. Each bubbler was filled with 50 mL ethyl acetate. Three 1-hour bubbler samples were taken each exposure day. From each impinger, 10-mL samples were taken and analyzed by gas chromatography (GC). The generation system was adjusted as needed on the basis of the previous day's GC data.

IN-LIFE OBSERVATIONS

Rats and mice were observed twice daily, once in the early morning and once in the afternoon, for dead or moribund animals. Moribund animals were euthanized and a necropsy performed. Each rat and mouse was weighed and observed for clinical signs of toxicity before BDO_2 exposures, 1 week after the exposures began, at the end of the exposures, and at monthly intervals thereafter.

DEATHS AND NECROPSIES

Scheduled Deaths

Animal deaths were scheduled as follows: 8 animals in each group (total: 24 mice and 24 rats) at the end of exposure, 4 animals in each group (total: 12 mice and 12 rats) at 6 months after exposure, 4 animals in each group (total: 12 mice and 12 rats) at 12 months after exposure, and 40 animals in each group (total: 120 rats and 120 mice) at the termination of the experiment (Table 1). Eight animals (4 rats and 4 mice) were killed at the end of the quarantine period but before the scheduled exposure. These schedules were modified because of the unscheduled deaths of a large number of rats. The scheduled deaths at the end of the exposures were completed for rats and mice, but only mice were killed at 6 months after exposure, and no animals were killed at 12 months.

At the time of necropsy, the animals were weighed and killed using pentobarbital (250 to 300 mg/kg) administered by i.p. injection. The chest cavity was opened immediately, and the animals exsanguinated by cardiac puncture using a syringe and needle. All animals, including those that died spontaneously or were euthanized, were given a complete necropsy including all organ systems. All lesions were noted on Individual Animal Necropsy Records designed specifically for this study. Tissues examined and saved in 10% neutral buffered formalin included adrenals, aorta, brain, clitoral gland, esophagus, eyes, femur, gall bladder (mice), Harderian gland, heart, intestine (duodenum, jejunum, ileum, cecum, colon, rectum), kidneys, lacrimal gland, larynx, liver, lungs, lymph nodes (bronchial, mesenteric), mammary gland, nose, ovaries, pancreas, parathyroid, pituitary gland, salivary gland, skin, spleen, stomach, thymus, thyroid gland, tongue, trachea, urinary bladder, uterus, vagina, vertebra, and Zymbal's gland.

Of these tissues, only target organs noted in the rat or mouse bioassays were routinely trimmed and sectioned for histologic examination. These included heart, lungs, stomach, liver, Harderian gland, ovaries, pancreas, uterus, thyroid gland, Zymbal's gland, mammary gland, and kidneys. In addition, any organs suspected of having an increased incidence of neoplasia based on gross necropsy were trimmed and sectioned.

Target tissues were fixed in 4% buffered paraformaldehyde overnight and transferred to 70% ethanol. Tissues for histopathological evaluation (including nose and left femur after decalcination) were trimmed, embedded in paraffin, sectioned at about 5-µm intervals, and stained with hematoxylin and eosin.

The nose was decalcified in formic acid solution and trimmed for paraffin embedding. Four cross sections of the nose were taken initially (Levels 2 to 5, Figure 1). Because

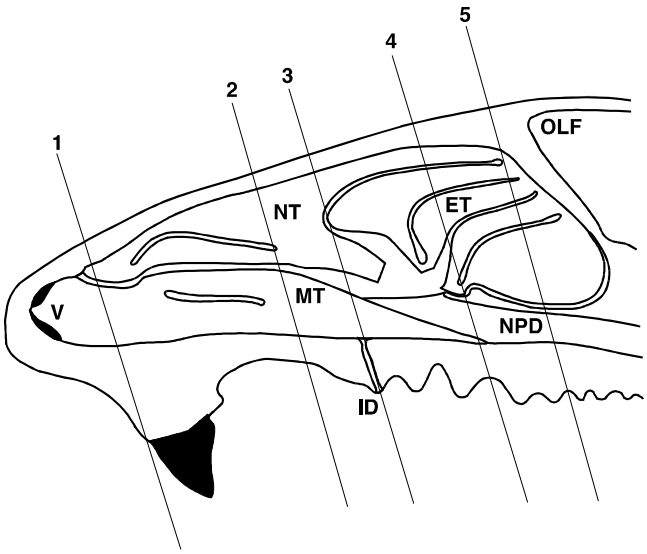

Figure 1. Cross sections of the nose taken for histologic evaluation. V = vestibule; NT = nasoturbinate; MT = maxilloturbinate; ID = incisive duct; ET = ethmoturbinate; NPD = nasopharyngeal duct; OLF = olfactory bulb.

extensive lesions were noted in the nasal mucosa of the rats, a fifth section was added (Level 1). Lesions noted histologically in the nasal mucosa were recorded on nose maps (Mery et al. 1994) to understand better the distribution of the lesions throughout the nasal cavity.

Although the Zymbal's gland was a protocol-designated tissue, it is not included in the tables of results because of the low sectioning rate and the lack of gross lesions in the gland and in the related clitoral gland. Routine sectioning of the Zymbal's gland resulted in a few good profiles, and they were normal.

K-*ras* Mutations

To determine if BDO_2-induced tumors contained mutations similar to those reported for BD-induced tumors (Goodrow et al. 1990), DNA was isolated from fixed tissue. The polymerase chain reaction (PCR) was used to amplify the first exon of the K-*ras* gene so that codon 13 mutations could be identified by direct sequencing. Tumors were microdissected and amplification of K-*ras* exon 1 attempted. Tissue sections (5 µm) containing nasal lesions were deparaffinized and stained with toluidine blue to facilitate microdissection. Microdissected tissue was digested overnight at 55°C in lysis buffer containing Tris-HCl (10 mM), potassium chloride (50 mM), magnesium chloride (2.5 mM), Tween 20 (0.45%), and proteinase K (500 µg/mL). In general, digestions were carried out in a final volume of 40 µL. Two separate aliquots (4 µL and 10 µL) were used in attempting to amplify the first exon of the K-*ras* gene. Primers and PCR conditions have been described (Nickell-Brady et al. 1994). The strategy for amplification was as follows. A first-round amplification of 35 cycles was conducted using primers that crossed both introns in order to avoid amplification of the K-*ras* pseudogene. Five microliters of PCR product from the first amplification was then reamplified for 35 cycles using a hemi-nested approach where a new 3′ primer was used with the original 5′ primer.

Noncancer Endpoints

Hematological parameters evaluated in blood obtained at the time of death included complete blood count, differential cell counting, hematocrit, and hemoglobin content.

STATISTICAL METHODS

Mice and rats were exposed to 0, 2.5, or 5.0 ppm BDO_2 for 6 weeks and subsequently held for 18 months. During this time, body weight was measured at approximately monthly intervals. Animals killed immediately after exposure and at the end of 18 months following exposure were examined for lesions. Selected hematologic parameters were also measured in these animals. The study design called for scheduled deaths at 6 and 12 months after exposure. Due to the number of spontaneous deaths, only the mice were killed at 6 months. The data for the interim deaths at 6 months following exposure are only included in selected statistical analyses. The statistical methods implemented to analyze these three types of data (body weight measurements, lesions, and hematologic parameters) are described in the following paragraphs. A general survival analysis was also performed. All analyses were performed separately for mice and rats. The statistical software used for the analyses was SAS.

SURVIVAL ANALYSIS

The survival distribution function was estimated for each stratum (BDO_2 exposure level) using the nonparametric Kaplan-Meier method. Two rank tests were used to evaluate homogeneity across all strata (0, 2.5, and 5.0 ppm BDO_2). The censored-data generalization of the Savage test, commonly called the log-rank test, places more weight on longer survival times, whereas the censored-data generalization of the Wilcoxon test places more weight on shorter survival times. Thus, comparison of the two tests can provide additional information on how survival distribution functions differ. If homogeneity across all strata was rejected ($p \leq 0.05$) by either rank test, pairwise comparisons were made. Because three comparisons were made (0 versus 2.5, 0 versus 5.0, and 2.5 versus 5.0), a Bonferroni-type adjustment was made so that, at the

0.05 level, statistical significance was accepted for $p \leq 0.017$. The animals killed at one of the early time points (i.e., immediately after exposure for the rats or, for the mice, immediately after exposure and at 6 months after exposure) were not included in this analysis.

BODY-WEIGHT ANALYSIS

Body-weight measurements were made prior to initiation of exposure, 1 week after the exposure began, at the end of the exposure, and at monthly intervals thereafter for 18 months. For mice, a multivariate repeated-measures procedure was performed to compare body weights among the three exposure groups at six identified time points: immediately prior to exposure (to test that all animals had similar body weights at the beginning of the experiment); at the end of the exposure (to test acute effects of the 6-week exposure); at 1, 6, and 12 months after the end of the exposure (to test any developing effects due to the exposure); and at the time of death. Statistical significance ($p \leq 0.05$) was assessed by the Hotelling-Lawley trace. The multivariate tests determined the next level of analysis. If there was a statistically significant interaction between the multivariate vector of dependent variables (body weight at the six time points) and the independent factor (BDO_2 exposure level) or if there were statistically significant effects for both the dependent-variable vector (i.e., statistically significant differences in body weight over time) and the independent factor, the next stage of the analysis was to examine the univariate analyses of variance (ANOVAs) for each dependent variable (i.e., six ANOVAs, one for each time point) for significance of the single factor, BDO_2 exposure level. A statistically significant factor ($p \leq 0.05$ evaluated with an F test) permitted comparison of the body weights in the different exposure groups at the single point in time. These comparisons were made with t tests. Due to the step-down nature of the analysis, no multiple-comparison corrections were made. If there was a statistically significant BDO_2 effect from the repeated-measures multivariate tests but no body weight–vector significance, the body weights were averaged and the data analyzed in a single one-way ANOVA. Subtesting of a significant BDO_2 effect was with an uncorrected t test. Finally, if there was a significant weight effect from the repeated-measures analysis but no BDO_2 effect, no further testing was performed as weight changes unassociated with exposure were not of interest for this study.

Repeated-measures (and all multivariate) techniques require that there be no missing data in the vector of response variables (e.g., no missing weights at any of the time points identified for this analysis). Thus, only mice that had been randomized into the groups for end-of-study death were included in this analysis. Additionally, a small number of mice were excluded from the analysis because they died before the end of the experiment. However, the sample sizes remained adequate (40, 42, and 36 in the 0, 2.5, and 5.0 ppm BDO_2 exposure groups, respectively) to perform the repeated-measures analysis as described above.

Because of the large number of early deaths in the rats, however, the repeated-measures approach could not be taken for the entire time period. For example, only two rats in the group exposed to 5.0 ppm BDO_2 survived to the end of the study. A repeated-measures analysis was used looking only at four time points: immediately prior to exposure, at the end of exposure, and at 1 and 6 months after exposure. These corresponded to relatively early time points in the study. The sample sizes for this analysis were 48, 45, and 35 for the rats exposed to 0, 2.5, and 5.0 ppm BDO_2, respectively.

HEMATOLOGIC DATA ANALYSIS

Fourteen hematologic parameters were measured: leukocytes (white blood cells), erythrocytes (red blood cells), hemoglobin, hematocrit, mean cell volume, platelets, and up to eight cell types reported as cell differentials (segmented neutrophils, lymphocytes, monocytes, eosinophils, band neutrophils, basophils, and immature cells [blasts, nucleated red blood cells]). The data were grouped for statistical treatment and analyzed in a step-down fashion. First, a vector of primary variables was identified. These variables were considered to have primary importance in identifying possible effects from the exposure. Four variables were in this vector: white blood cells, red blood cells, platelets, and mean cell volume. These variables formed the dependent-variable vector of the primary-variable multivariate analysis of variance (MANOVA); the independent variables were exposure concentration (0, 2.5, or 5.0 ppm BDO_2) and time of death (6 weeks, 18 months).

If statistically significant effects ($p \leq 0.05$, Hotelling-Lawley trace) due to concentration or the concentration × time interaction were observed, then the ANOVAs for each primary variable were examined. Statistical significance was evaluated using the factor F tests ($p \leq 0.05$) for the concentration factor and the concentration × time interaction. Subtesting of statistically significant effects was performed using uncorrected t tests. If there was an ANOVA concentration effect but no concentration × time interaction, then the t tests were performed on the concentration factor means (i.e., averaged over the two time periods).

If the primary variable red blood cell count had a statistically significant effect due to concentration or the interaction between concentration and time, then a second MANOVA

was performed. In this MANOVA, the vector of dependent variables consisted of hemoglobin and hematocrit, both of which would provide supporting information elaborating on the results of the red blood cell count. As described above for the primary variables, significance at the MANOVA level permitted examination of the individual ANOVAs; significance at the ANOVA level resulted in uncorrected *t* tests of the means or factor means, as appropriate.

A MANOVA was run for the cell differentials, regardless of the statistical significance of the primary-variable MANOVA or the white blood cell ANOVA. This is because it would be possible for the total cell counts to be unaffected by treatment but for the differentials to shift. Because the differentials sum to 100%, the monocytes were excluded from the MANOVA and analyzed in a separate ANOVA. The testing for the remaining cell differentials in the MANOVA proceeded as described earlier. Before examining the ANOVAs, multivariate significance was required. Before examining the uncorrected *t* tests, significance of the concentration factor or the concentration × time interaction was required.

ANALYSIS OF NEOPLASTIC LESIONS

Due to the extensive spontaneous deaths in the rats, the statistical approach for analyzing the lesion data is different from that used for the mice. In the rats, a survival analysis was performed using only those animals that were found dead or found moribund and euthanized. The analysis followed the techniques described earlier for the general survival analysis. The results reported, however, are only the median survival time and its standard error.

The neoplastic lesion data for the mice were analyzed using contingency tables. Only those animals that survived until end-of-study deaths were included in this statistical analysis. Lesions were observed in several tissues although generally there was only one lesion in any mouse. There was no particular tissue for which it was more likely to observe lesions, in contrast to the rats, in which all lesions were observed in nasal tissue. As a result, the contingency tables were constructed four ways. First, the mice were classified according to whether they had any lesion in any tissue or no lesions at all. Second, on the basis of findings in historical studies, the mice were classified on whether they had any lesion in the reproductive organs or not. Third, on the basis of both historical information and the observations from the rats, the mice were classified on whether they had any lesion in the respiratory region or not. Finally, a single contingency table was constructed to test for the effects of BDO_2 exposure on the Harderian gland because this gland is in the eye and would receive direct exposure to BDO_2 in the nose-only exposure system.

Because the contingency tables had three rows, corresponding to the three dose levels of BDO_2 (0, 2.5, or 5.0 ppm), a significant chi-square test ($p \leq 0.05$) was subtested by constructing the 2 × 2 contingency tables. The chi-squares from these tests were adjusted for multiple comparisons so that statistical significance was considered to be observed when $p \leq 0.017$.

RESULTS

EXPOSURES

The mean ± SD of the BDO_2 concentrations in the three chambers were 0 (control), 2.48 ± 0.42 (low level), and 5.07 ± 0.65 (high level) ppm. The mean concentrations were within 1.5% of the target values. The coefficients of variation were less than 20%.

IN-LIFE OBSERVATIONS

Clinical Observations

The predominant clinical findings attributable to BDO_2 exposure in rats and mice were due to lesions in the upper respiratory tract. Rats inhaling 2.5 and 5.0 ppm BDO_2 developed a broad spectrum of clinical signs associated with obstruction of the upper airways and, unlike mice, these signs occurred only after the BDO_2 exposures had ended. The major clinical sign was dyspnea, which was first observed approximately 4 weeks after termination of exposures. The incidence peaked in 2.5 ppm– and 5.0 ppm–exposed rats approximately 12 and 8 weeks after exposure, respectively, and remained elevated in both groups until approximately 24 weeks after exposure. The time course of development of this clinical symptom in rats is illustrated in Figure 2.

Wheezing and, to a lesser extent, tachypnea were observed in rats inhaling 5.0 ppm BDO_2. Wheezing occurred in approximately 20% of these between 4 and 20 weeks after exposure. Tachypnea occurred in less than 3% of the rats between 4 and 34 weeks after exposure. Abdomens of rats inhaling 2.5 and 5.0 ppm BDO_2 were periodically swollen due to their swallowing air. The time course for development of swollen abdomens in each dose group paralleled that of dyspnea. The obstruction of the upper airways prevented eating, leading to weight loss prior to death or moribund euthanasia.

No control mice or mice exposed to 2.5 ppm BDO_2 displayed clinical signs of toxicity. All mice exposed to 5.0 ppm

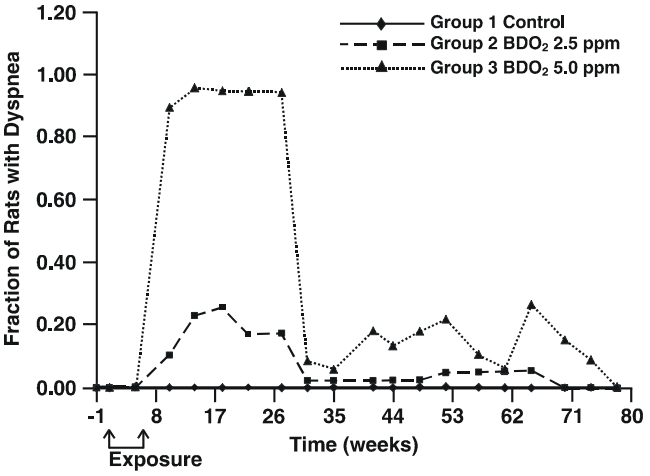

Figure 2. Time course of dyspnea in rats.

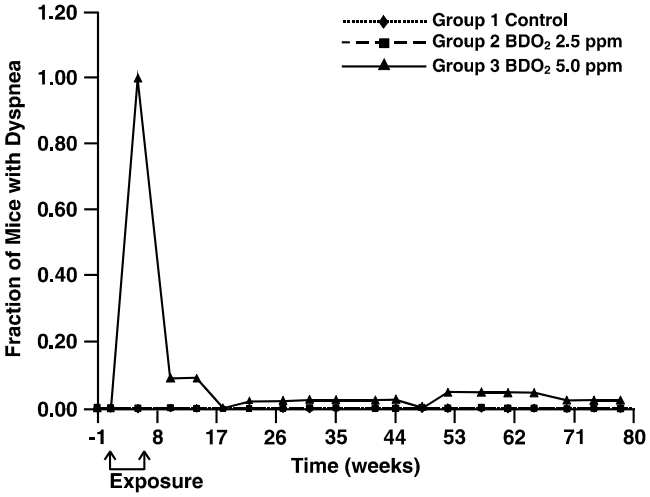

Figure 3. Time course of dyspnea in mice.

BDO_2 had dyspnea by exposure week 5, but the prevalence dropped to less than 10% following termination of exposures. The time course for occurrence of dyspnea in mice is illustrated in Figure 3.

Body Weight

The mean monthly body weights of the rats and mice are shown in Figures 4 and 5, respectively. Throughout the post-exposure period, the rats exposed to 5.0 ppm BDO_2 weighed approximately 20% less than the control rats. Initially, an approximate 20% reduction in body weight was observed in mice exposed to the high level of BDO_2.

The repeated-measures analysis of body weight in the rats (time points correspond to weeks 1, 6, 10, and 30 on Figure 4) detected a statistically significant interaction ($p < 0.01$) between the body-weight vector and BDO_2 exposure level. The results of the comparisons at each time point indicated no statistically significant difference among the rats assigned to the three exposure levels prior to the initiation of exposure. By the end of the exposure, the body weights of the rats exposed to 5.0 ppm BDO_2 were significantly depressed ($p < 0.01$) relative to the other two exposure groups. The body weights of the rats in the high-dose exposure group remained depressed relative to the two other groups at 10 and 30 weeks after exposure. The body weights of rats exposed to 2.5 ppm BDO_2 were lower than the body weights of the control rats. At 10 weeks, this difference was statistically significant ($p = 0.04$) but did not attain statistical significance at the other time points ($p = 0.08$ at 6 weeks; $p = 0.29$ at 30 weeks).

The repeated-measures analysis of body weight in the mice (time points correspond to weeks 1, 6, 10, 30, and 56 on Figure 5; weight at death is not included in the figure) detected a statistically significant interaction ($p < 0.01$)

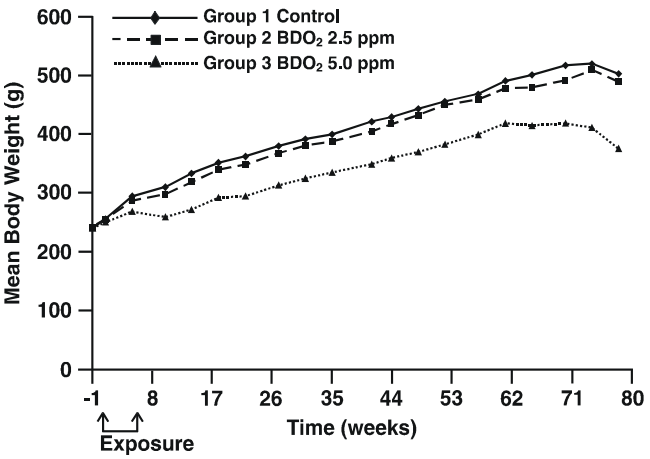

Figure 4. Mean body weights of rats exposed for 6 weeks.

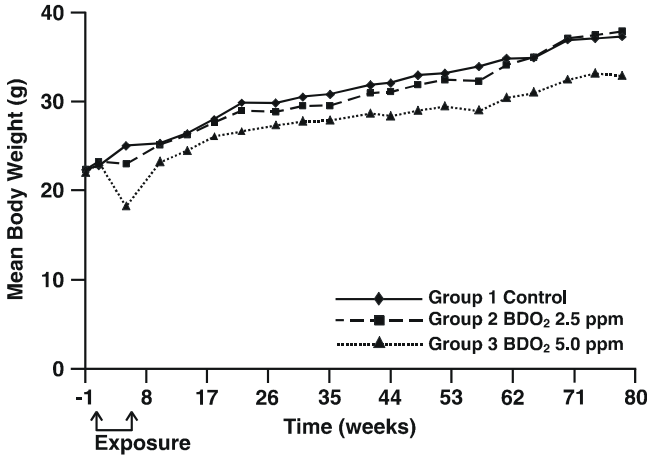

Figure 5. Mean body weights of mice exposed for 6 weeks.

between the body-weight vector and BDO_2 exposure level. The results of the comparisons at each time point indicated statistically significant differences among the mice assigned to the three exposure levels at all time periods. Prior to exposure, the average (± SE) body weight of the control mice was 22.5 ± 0.1 g. Although this value was statistically different from the average body weight of the mice in the 5.0 ppm BDO_2 exposure group (20.0 ± 0.1 g), this difference is not biologically meaningful. At the end of exposure, the mice exposed to 5.0 ppm BDO_2 lost weight and weighed, on average, 6.6 g less than the control mice. In subsequent periods, the mice exposed to the high concentration of BDO_2 weighed between approximately 2 and 4.5 g less than the control mice. The weights of the mice exposed to 2.5 ppm BDO_2 were generally within 1 to 1.5 g of the control mice. Immediately following exposure, however, the weights in the 2.5 ppm BDO_2 exposure group were 2 g lower than the weights of the control mice. These differences were statistically significant at some periods (6, 30, and 56 weeks) but not at the other time points.

Survival

The fates of rats and mice in each exposure group are shown in Tables 2 and 3, respectively. Survival curves for all groups are shown in Figure 6 (rats) and Figure 7 (mice). The control rats did not survive as long as the mean survival of female Sprague-Dawley rats in 15 2-year studies as published by the supplier (Charles River Laboratories 1992) and shown in Figure 8. However, the survival of both rats (log-rank and Wilcoxon, $p < 0.01$) and mice (log-rank, $p = 0.05$; Wilcoxon, $p = 0.03$) differed by exposure group with the control animals outliving the treated animals.

No rats died during the exposure, but 13 rats (12 exposed to 5.0 ppm, 1 exposed to 2.5 ppm) died within 3 months after the exposure ended. Deaths were due to clogged nasal passages caused by necrosis, inflammation, and squamous metaplasia of the nasal mucosa. As shown in Table 4a and illustrated in Figure 6, exposure to 5.0 ppm BDO_2 was associated with significantly reduced survival relative to either control air or 2.5 ppm BDO_2. The decrease in survival in rats exposed to 2.5 ppm BDO_2 was not significantly different from control when the Bonferroni adjustment was made.

Four of the mice exposed to the high level of BDO_2 died during the last week of exposure due to blocked nasal passages. After the end of the exposure, survival of mice was good in all groups. As indicated by the pair-wise comparisons with a Bonferroni correction (Table 4b), there were no statistically significant differences.

Table 2. Fate of Rats by Exposure Group

	BDO_2 Exposure Group[a] (ppm)		
	0	2.5	5.0
Died during exposure	0	0	0
Killed at end of exposure	8	8	8
Found moribund and euthanized	31	37	46[b]
Killed 18 months after end of exposure	17	11	2
Unscheduled deaths after end of exposure (% of animals at risk)	65%	77%	94%

[a] $n = 56$ for each exposure concentration.

[b] Includes one accidental death.

Table 3. Fate of Mice by Exposure Group

	BDO_2 Exposure Group[a] (ppm)		
	0	2.5	5.0
Died during exposure	0	0	4
Killed at end of exposure	8	8	8
Killed 6 months after end of exposure	4	4	3
Found moribund and euthanized	4	2	5
Killed 18 months after end of exposure	40	42	36
Unscheduled deaths after end of exposure (% of animals at risk)	9.1%	4.5%	11%

[a] $n = 56$ for each exposure concentration.

Viral Screens

Viral screens of rats and mice before the start of exposures were negative.

OBSERVATIONS AT DEATH

Hematology

Hematological parameters were measured at the end of the exposure and at the end-of-study deaths. The statistical analyses were performed following a step-down procedure and are shown in Table 5 (rats) and Table 6 (mice). Selected variables are graphed for the rats at 6 weeks (Figure 9) and

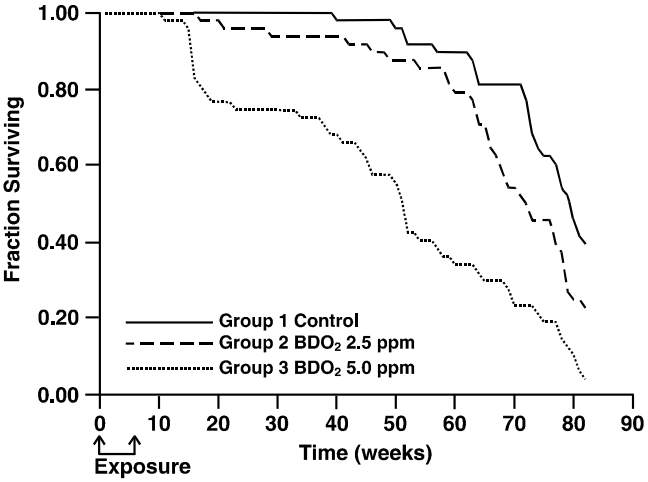

Figure 6. Survival curves for rats held for long-term observation following a 6-week exposure. There was no statistically significant difference in overall survival of rats exposed to clean air or 2.5 ppm BDO_2. Survival was decreased in rats exposed to 5.0 ppm relative to the other two groups.

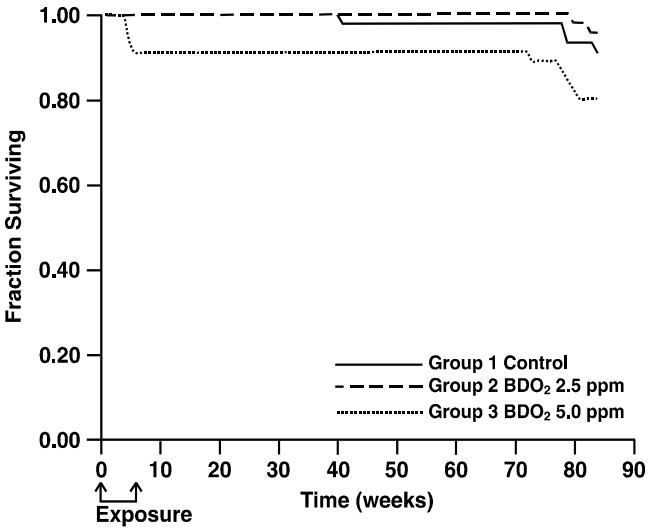

Figure 7. Survival curves for mice held for long-term observation following a 6-week exposure. There was no statistically significant difference for any pairwise comparisons of the three groups.

at 18 months (Figure 10) and for mice at 6 weeks (Figure 11) and at 18 months (Figure 12).

The exposure effects found in the rats were related to red blood cells and the two supporting variables, the hematocrit and hemoglobin. For all three variables, a significant exposure effect was found ($p \leq 0.05$). The factor means (averaged over the two time periods of 6 weeks and 18 months), shown in Table 7, increased with exposure to BDO_2 but not in a dose-dependent manner. The increases would not be biologically significant. Although there were no statistically significant concentration differences in the cell differential MANOVA, there was a statistically significant concentration effect in the excluded variable, monocytes. Monocytes decreased following exposure to BDO_2 but did not decrease in a dose-dependent fashion (Table 7).

The time effects found in the primary variables for the rats were a statistically significant increase in mean cell volume and platelets between 6 and 80 weeks after exposure and a statistically significant decrease in both red and

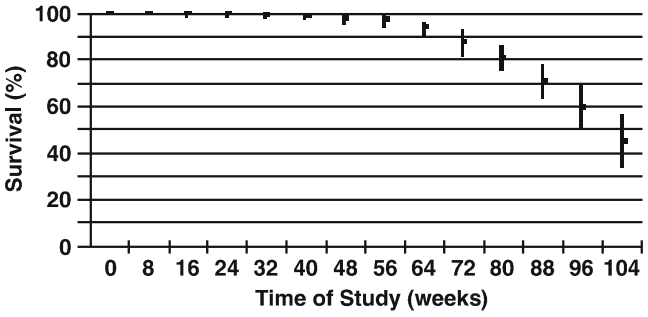

Figure 8. Mean survival rates of female Sprague-Dawley rats in 15 two-year studies (Charles River Laboratories 1992).

Table 4a. Survival of Rats and Mice[a]

BDO_2 (ppm)	Rats	Mice
0	519.9 ± 10.4	579.6 ± 7.8
2.5	479.3 ± 16.1	580.5 ± 0.8
5.0	349.1 ± 24.4	516.1 ± 23.8

[a] Values give mean ± SE survival times in days.

Table 4b. Statistical Significance from the Analyses of Survival Data[a]

BDO_2 (ppm) Group Comparison	Log-Rank	Wilcoxon
Rats		
All groups	< 0.01[b]	< 0.01[b]
0 vs. 2.5	0.070	0.045
0 vs. 5.0	< 0.001[b]	< 0.001[b]
2.5 vs. 5.0	< 0.001[b]	< 0.001[b]
Mice		
All groups	0.05[b]	0.03[b]
0 vs. 2.5	0.378	0.448
0 vs. 5.0	0.133	0.090
2.5 vs. 5.0	0.024	0.022

[a] Data are p values from the log-rank test, which places more weight on longer survival times, or the Wilcoxon test, which places more weight on shorter survival times.

[b] Statistically significant group comparison ($p \leq 0.05$). For the multiple pair-wise comparisons, the nominal p value (shown in the table) corresponding to statistical significance is 0.017.

Table 5. Statistical Significance from the Analyses of the Rat Hematologic Data[a]

Variable	Statistical Analysis	Time	Concentration	Time × Concentration
Primary	Multivariate	< 0.01*	0.02*	0.20
Mean cell volume	Univariate	< 0.01*	0.25	0.22
Platelets		< 0.01*	0.39	0.15
Red blood cells		< 0.01*	< 0.01*	0.20
White blood cells		0.04*	0.61	0.72
Red blood cell supporting	Multivariate	< 0.01*	0.05*	0.62
Hemoglobin	Univariate	0.27	0.04*	0.55
Hematocrit		< 0.01*	0.01*	0.41
Cell differentials	Multivariate	< 0.01*	0.57	0.86
Segmented neutrophils	Univariate	< 0.01*	0.73	0.81
Lymphocytes		< 0.01*	0.91	0.43
Band neutrophils		0.51	0.80	0.80
Eosinophils		0.37	0.37	0.79
Basophils		0.37	0.69	0.69
Monocytes	Univariate	0.30	0.05*	0.15

[a] Data are p values, which are significant if $p < 0.05$. Multivariate significance was determined from the Hotelling-Lawley trace. Those effects that permitted the next level of analysis (i.e., the ANOVAs) are indicated with an asterisk.

Table 6. Statistical Significance from the Analyses of the Mouse Hematologic Data[a]

Variable	Statistical Analysis	Time	Concentration	Time × Concentration
Primary	Multivariate	< 0.01*	0.36	0.06
Mean cell volume	Univariate	< 0.01*	0.66	0.08
Platelets		< 0.01*	0.40	0.60
Red blood cells		< 0.01*	0.06	0.58
White blood cells		0.27	0.37	0.12
Red blood cell supporting	Multivariate	< 0.01*	0.12	0.56
Hemoglobin	Univariate	< 0.01*	0.43	0.94
Hematocrit		< 0.01*	0.13	0.68
Cell differentials	Multivariate	< 0.01*	< 0.01*	0.74
Segmented neutrophils	Univariate	< 0.01*	< 0.01*	0.13
Lymphocytes		< 0.01*	< 0.01*	0.11
Band neutrophils		0.27	0.94	0.94
Eosinophils		0.07	0.84	0.61
Basophils		0.03*	0.47	0.47
Immature cells (blasts)		0.54	0.89	0.89
Monocytes	Univariate	< 0.01*	0.86	0.75

[a] Data are p values, which are significant if $p < 0.05$. Multivariate significance was determined from the Hotelling-Lawley trace. Those effects that permitted the next level of analysis (i.e., the ANOVAs) are indicated with an asterisk.

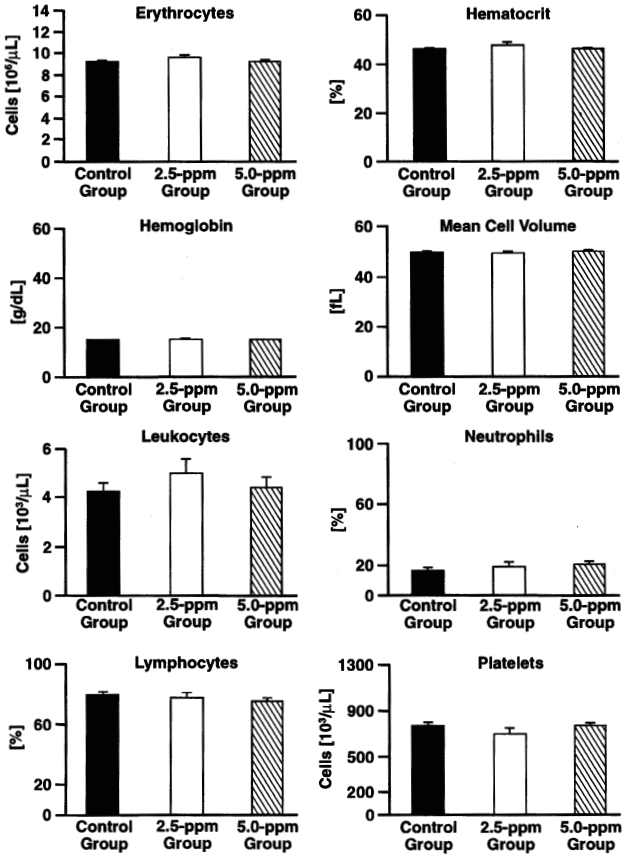

Figure 9. Hematologic parameters observed in rats at the end of the 6-week exposure. Bars show means and error bars.

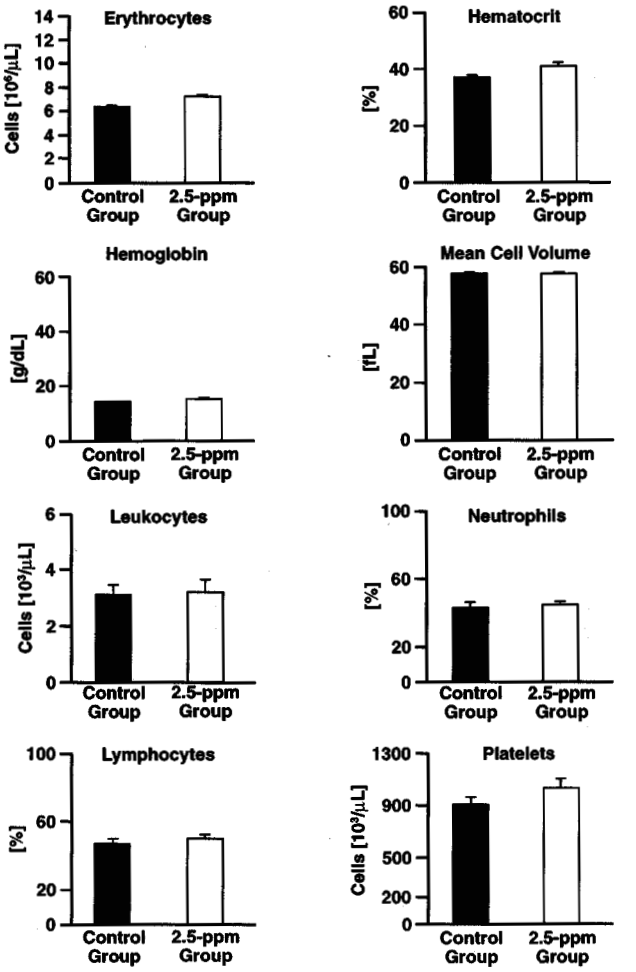

Figure 10. Hematologic parameters observed in rats at the end of study, 18 months after the end of exposure. Only two rats from the group exposed to 5.0 ppm BDO_2 survived to 18 months and an adequate blood sample was obtained from only one of these animals; therefore, those data are not reported. Bars show means and error bars.

white blood cell counts over the same time period (observed in all exposure groups, including controls). Consistent with the decrease in red blood cell counts, hematocrit also decreased significantly. Hemoglobin dropped slightly between 6 and 80 weeks, but this difference was minimal and not statistically significant. The time effects observed in the cell differentials were found to be attributable to a substantial increase in the segmented neutrophils that was offset by an approximately comparable decrease in lymphocytes, which occurred in all exposure groups, including the controls. No interaction between time and concentration effects was noted.

Statistically significant concentration effects in mice were observed in the cell-differential variables (see Table 6). Segmented neutrophils increased significantly in a dose-dependent fashion, whereas lymphocytes decreased in a dose-dependent fashion (Table 8).

As for the rats, mean cell volume and platelets increased significantly between 6 and 80 weeks, while red blood cell counts decreased. White blood cells also decreased; however, the difference was not statistically significant. The time-related decreases in both hemoglobin and hematocrit were statistically significant. The time-related increase in the percentage of segmented neutrophils combined with the increase in basophils was offset by the statistically significant decrease in lymphocytes. Statistically significant concentration effects were also observed in both the segmented neutrophils and the lymphocytes. These concentration effects were parallel (i.e., there was no concentration × time interaction), so that the effect did not worsen (or was not ameliorated) over time.

K-*ras* Activation

Following the end-of-study deaths, DNA was isolated from the upper respiratory tract tumors, and PCR techniques were used to amplify the first exon of the K-*ras* gene. The only PCR product resolved on the agarose gel was that of the positive control, which was normal lung.

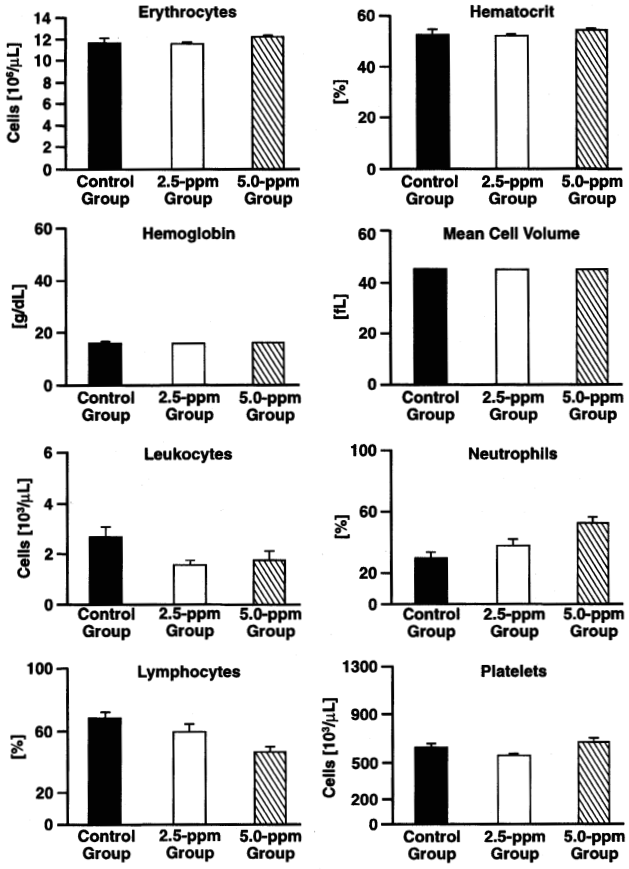

Figure 11. Hematologic parameters observed in mice at the end of the 6-week exposure. Bars show means and error bars.

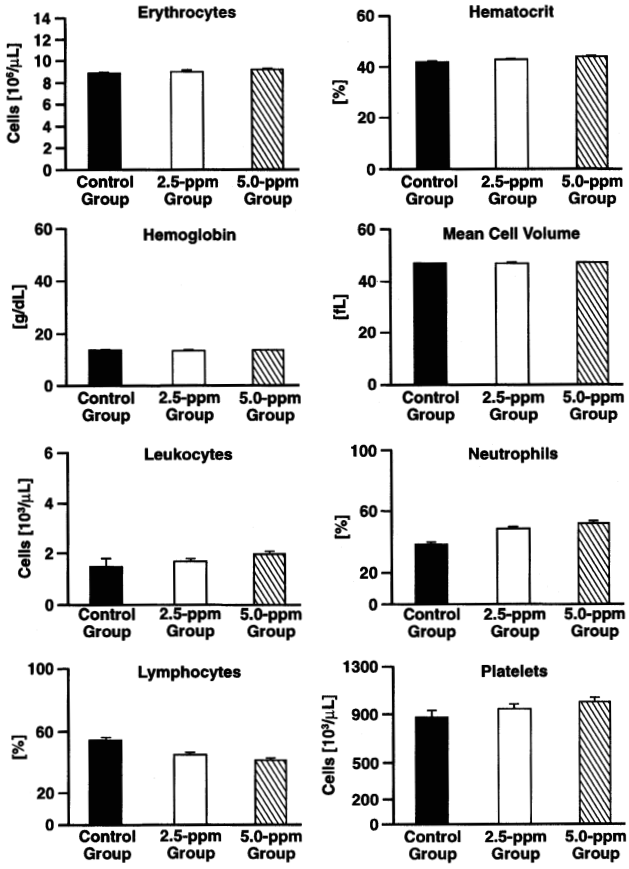

Figure 12. Hematologic parameters observed in mice at the end of study 18 months after the end of exposure. Bars show means and error bars.

The inability to amplify DNA from the nasal tumors most likely stems from the fact that the noses were decalcified in 13% formic acid for 3 to 4 days prior to embedding and sectioning. While this facilitates morphologic examination, it also hydrolyzes the DNA, apparently into fragments too small to amplify.

Histopathology of Rats

Nonneoplastic Lesions The only exposure-related, nonneoplastic lesions were in the nasal mucosa. These lesions were centered around the main airflow pathway through the nose. The distributions of the lesions are noted in Figures 13 and 14. The extent and severity of the lesions varied with dose and time after exposure.

In the rats killed at the end of exposure, lesions were found only in the nose. In the rats exposed to 5.0 ppm, the lesions were characterized by necrosis, inflammation, squamous metaplasia of the mucosa lining the anterior nasal cavity, and atrophy of the underlying maxilloturbinate and nasoturbinate bones (Table 9; Figure 15). Fibrinopurulent exudate and necrotic debris were present in the nasal cavity. In addition, there was acute inflammation with ulceration of the mucosa lining the nasopharyngeal duct. Necrosis of the anterior reaches of the olfactory epithelium lining the dorsal meatus and atrophy of the vomeronasal organ were present in a few rats. In rats exposed to 2.5 ppm, the lesions were similar, but much less severe and frequent. Inflammation was prominent except in the nasopharyngeal mucosa, and necrosis and squamous metaplasia were infrequent in all areas of the nasal mucosa.

In rats that were found moribund and euthanized or that died after the end of the 6-week exposure, marked squamous metaplasia and chronic inflammation characterized the nasal lesions and involved primarily the transitional and respiratory epithelia (see Table 9; Figure 16). The lesions were more extensive and severe in the rats exposed to 5.0 ppm than in rats exposed to 2.5 ppm BDO_2 and more severe in rats that had unscheduled deaths than in rats killed at the end of exposure. Hyperkeratosis was prominent in the more severe metaplastic lesions. Large amounts of keratin debris were sloughed into the lumen of the nasal

Table 7. Rat Hematologic Data[a]

Variable	BDO_2 (ppm)	Percentage of White Blood Cells[b]	n[c]
Red blood cells	0	7.84 ± 0.11	21
	2.5	8.42 ± 0.11[d]	19
	5.0	8.41 ± 0.26[d]	9
Hemoglobin	0	14.7 ± 0.2	22
	2.5	15.4 ± 0.2[d]	19
	5.0	15.2 ± 0.4	9
Hematocrit	0	41.5 ± 0.6	22
	2.5	44.4 ± 0.6[d]	19
	5.0	43.6 ± 1.5	9
Monocytes	0	5.3 ± 0.5	22
	2.5	3.4 ± 0.6[d]	19
	5.0	3.4 ± 1.3	9

[a] Average of the 6-week and 18-month values.

[b] Least-squares means ± SE.

[c] Number of animals.

[d] Statistically significant ($p < 0.05$) difference from control exposure group (0 ppm BDO_2) as determined by uncorrected t tests.

Table 8. Mouse Hematologic Data[a]

Variable	BDO_2 (ppm)	Percentage of White Blood Cells[b]	n[c]
Segmented neutrophils	0	34.1 ± 1.9	46
	2.5	43.3 ± 1.9[d]	50
	5.0	52.5 ± 2.0[d]	44
Lymphocytes	0	61.1 ± 1.9	46
	2.5	52.3 ± 1.9[d]	50
	5.0	44.0 ± 1.9[d]	44

[a] Average of the 6-week and 18-month values.

[b] Least-squares means ± SE.

[c] Number of animals.

[d] Statistically significant ($p < 0.05$) difference from control exposure group (0 ppm BDO_2) as determined by uncorrected t tests.

cavity. In the nasopharyngeal duct, the debris occasionally occluded the air passage, a life-threatening event in an obligate nose-breathing species, such as the rat (Figure 17). Four rats had acute bronchopneumonia caused by aspiration of foreign material. The aspiration was most likely secondary to labored breathing caused by obstruction of the nasopharyngeal duct. Cyst formation was not a prominent finding in the study. In only one animal did a keratin squamous cyst develop in these areas of hyperkeratosis.

The inflammation was chronic and active, characterized by infiltration of lymphoid and monocytic cells accompanied by neutrophils and exudate in the lumen of the nasal cavity. The inflammation was most prominent in the mucosa lining the lateral walls and the nasal septum at the middle portion of the nasal cavity. Atrophy of the maxilloand nasoturbinates was frequent due to loss of the turbinate bones. Secretory metaplasia was occasionally seen in the respiratory epithelium, uncorrelated with other lesions. In the vestibule, normally lined by squamous or transitional epithelium, there were metaplasia and hyperkeratosis.

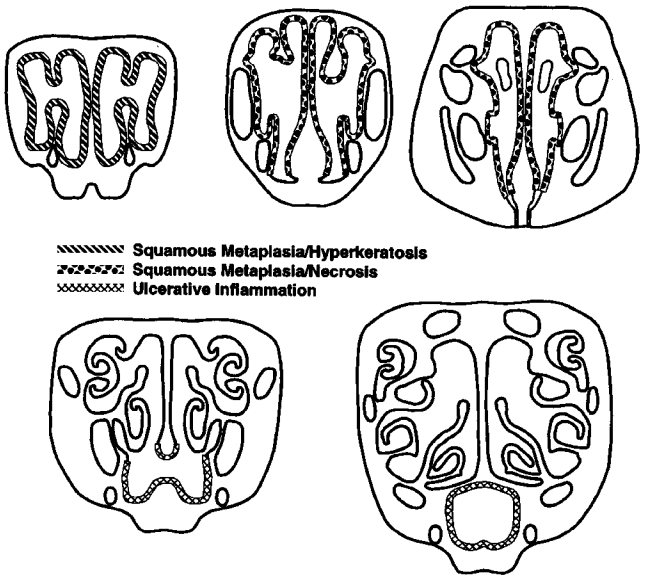

Figure 13. Distribution of nasal lesions in rats at the end of exposure to BDO_2.

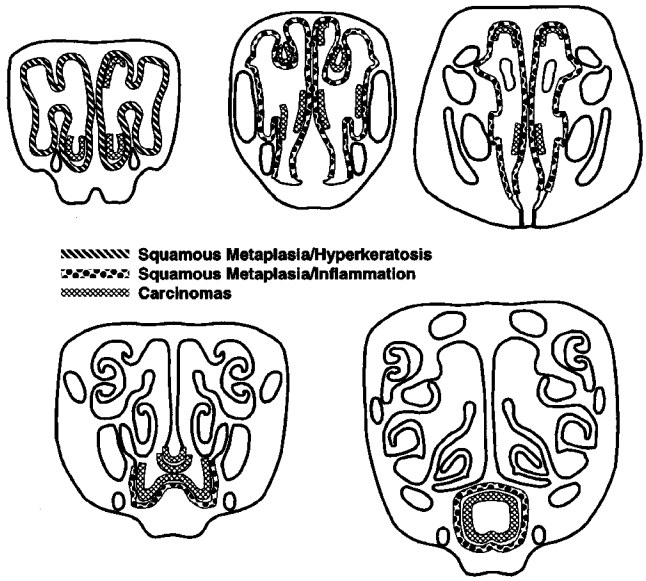

Figure 14. Distribution of nasal lesions in rats more than 60 days after the end of exposure to BDO_2.

The olfactory mucosa was not affected except in an occasional rat with mucoid secretory metaplasia. This metaplasia appeared to be secondary to marked squamous metaplasia or invasive, squamous cell carcinomas of the nasopharyngeal duct.

The severity of the metaplastic and inflammatory lesions depended on the exposure concentration and the time after the cessation of exposure. Inflammation, accompanied by necrosis, was most prominent in rats killed at the end of exposure.

A dose response was apparent for the inflammatory and metaplastic lesions. They were more severe and extensive at the high concentration (see Table 9). Moreover, after the end of the exposure period, these lesions persisted for the length of the study with a nearly consistent intensity. It was not easy to differentiate the morphologic appearance of lesions of those animals that died 300 days after exposure from those that died 582 days after exposure.

Neoplasia The only significant increase in neoplasia in exposed rats was in the nasal mucosa. Of the 48 rats held for long-term study in the 5.0 ppm group, 21 had neoplasms of the nasal mucosa. In the 2.5 ppm group, 12 of 48 rats had similar neoplasms (Table 10). Table 11 shows all 36 of the neoplasms of the nasal mucosa. Two rats in the high-exposure group had two malignant neoplasms each (I116 and I158), and one rat had a benign and a malignant neoplasm (I117).

Of the 12 rats with neoplasms following exposure to 2.5 ppm BDO_2, 8 deaths were attributed to the lesions. Since there were 37 unscheduled deaths in this exposure group, 22% of the mortality was attributable to fatal neoplasms. The mean (± SE) survival time of these 8 rats was 403 ± 43 days. In contrast, the mean survival time of all 48 rats exposed to 2.5 ppm BDO_2 was 479 ± 16 days. A similar set of calculations is shown for rats exposed to 5.0 ppm BDO_2. Because no fatal neoplasms were detected in control rats, the mean survival time of rats with fatal lesions was not calculated. The number of rats with neoplastic lesions following exposure to 5.0 ppm BDO_2 (44%) was nearly double that of those rats exposed to half the concentration. The percentage of fatal tumors, however, was relatively constant in the two groups (22% and 26%). The mean survival times (as well as the survival-function distributions) were not statistically significantly different between these two groups. The mean survival times for all rats in each exposure group are shown in Table 10 for reference. As described in Table 4 for all rats, survival time was significantly decreased with each increase in exposure concentration. Table 10 suggests that fatality due to neoplastic lesions was an important component of the decreased survival in rats exposed to 2.5 ppm BDO_2 (mean

Table 9. Prevalence of Nonneoplastic Lesions in Nasal Mucosa of Rats Exposed to BDO_2

	BDO_2 Exposure Group (ppm)		
	0	2.5	5.0
Deaths at End of Exposure			
Number of animals examined	8	8	8
Necrosis of nasal mucosa	0	38%	100%
Inflammation of nasal mucosa	0	63%	63%
Squamous metaplasia of nasal mucosa	0	25%	88%
Atrophy of turbinates	0	75%	100%
Unscheduled Deaths			
Number of animals examined	30	37	46
Necrosis of nasal mucosa	0	0	11%
Inflammation of nasal mucosa	6.6%	84%	85%
Squamous metaplasia of nasal mucosa	0	92%	100%
Atrophy of turbinates	0	89%	93%
End-of-Study Deaths			
Number of animals examined	17	11	2
Necrosis of nasal mucosa	0	0	0
Inflammation of nasal mucosa	24%	100%	100%
Squamous metaplasia of nasal mucosa	0	100%	100%
Atrophy of turbinates	0	100%	100%

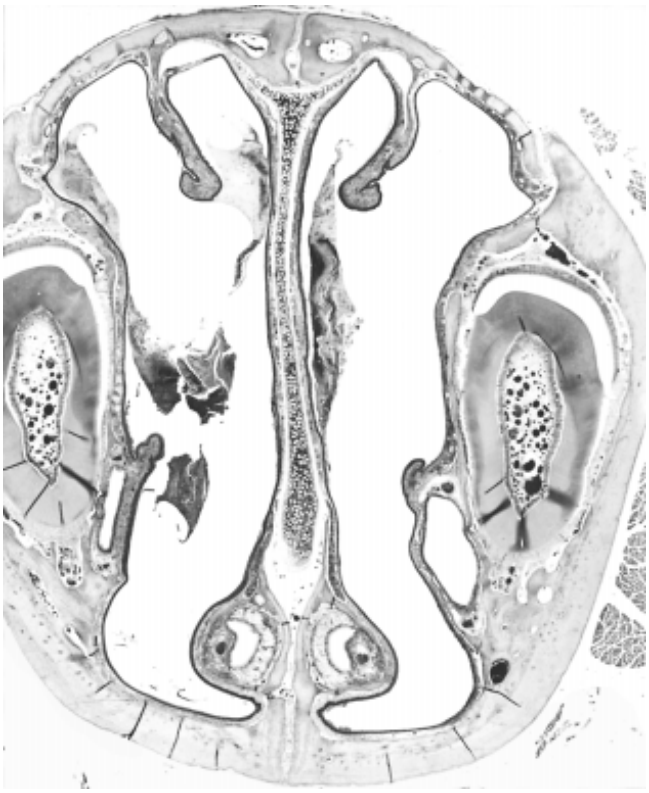

Figure 15. Photomicrograph of the cross section of the nasal cavity of a rat (I157) exposed to 5.0 ppm BDO_2 that died 26 days after the end of exposure. Note the atrophy of the naso- and maxilloturbinates and inflammatory exudate in nasal cavity. Magnification × 15; hematoxylin and eosin stain.

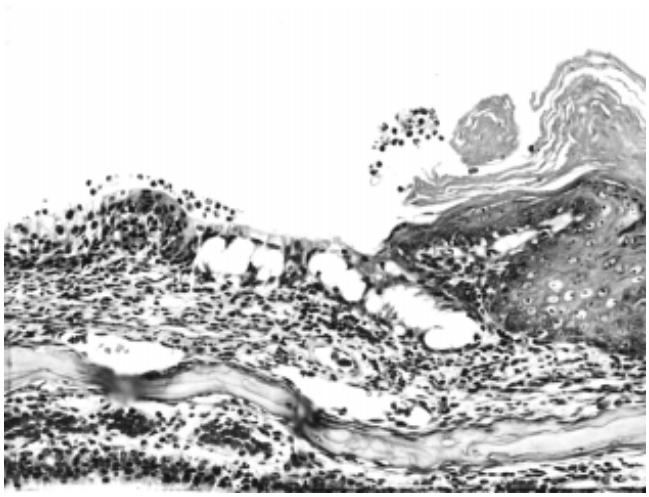

Figure 16. Photomicrograph of marked squamous metaplasia and chronic inflammation of the respiratory epithelium lining the nasopharyngeal duct in a rat (I125) 354 days after exposure to 5.0 ppm BDO_2. Magnification × 240; hematoxylin and eosin stain.

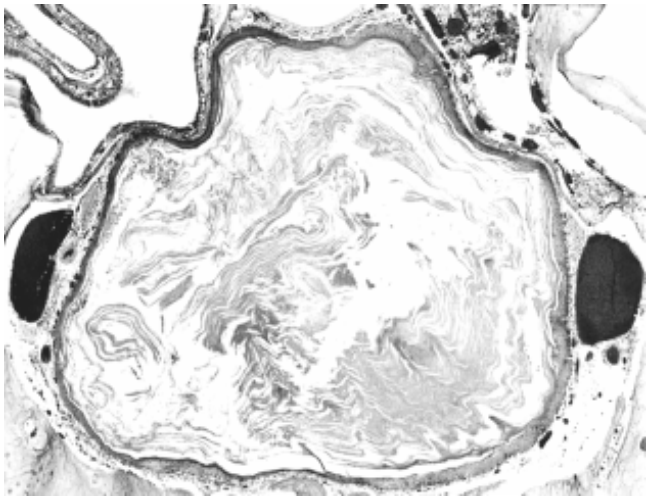

Figure 17. Photomicrograph of marked squamous metaplasia with accumulation of keratin in the nasopharyngeal duct in a rat (I160) 75 days after exposure to 5.0 ppm BDO_2. Magnification × 40; hematoxylin and eosin stain.

survival in rats with fatal lesions was 403 days compared to median survival in all rats of 479 days). In rats exposed to 5.0 ppm BDO_2, however, other causes of death contributed more to decreased mean survival than did fatal neoplasms.

The vast majority of the neoplasms arose in the respiratory epithelium of the nasal mucosa (see Table 11 and Figure 14). Of the mucosal sites that could be identified, 23 of 27 were respiratory epithelium. Several sites of origin within the nasal cavity were identified: the epithelium lining the vestibule, the septum, the lateral wall, the nasoturbinate, and the nasopharyngeal duct. In 14 of the cases the site of origin was the nasopharyngeal duct, and in 7 cases it was the septum; 3 tumors arose in the vestibule and 3 in the nasoturbinates or lateral wall. In 9 cases the site of origin could not be identified with certainty but did not involve the nasopharyngeal duct. Thus, the tumors were distributed throughout the nasal mucosa, where there was respiratory epithelium along the main airflow pathway (Figure 18).

The first death with a neoplasm of the nasal mucosa was 144 days after initiation of exposure and 102 days after the cessation of exposure. The neoplasm was a squamous cell carcinoma of the epithelium surrounding the nasopharyngeal duct. The neoplasm had occluded the duct, resulting in the death. This site was the most frequent in rats that died within 9 months after exposure (Figure 19). Sites anterior in the nasal cavity (epithelium lining the septum, lateral wall, and nasoturbinates) dominated at longer times after exposure.

The most common neoplastic lesion was squamous cell carcinoma (see Table 11). The carcinomas were associated with squamous metaplasia and chronic inflammation of the

Table 10. Prevalence of Metaplastic and Neoplastic Lesions in Nasal Mucosa of Rats Exposed to BDO_2

	BDO_2 Exposure Group[a] (ppm)		
	0 (n = 47)	2.5 (n = 48)	5.0 (n = 48)
Primary neoplasms, total	0	12	24
Rats with one or more, number	0	12	21
Rats with one or more	0	25%	44%
Benign neoplasms, total	0	0	1
Squamous papilloma	0	0	4.2%
Malignant neoplasms, total	0	12	23
Squamous cell carcinoma	0	23%	44%
Adenocarcinoma	0	0	4.2%
Sarcoma	0	4.2%	4.2%
Squamous metaplasia, total	0	45	48
Squamous metaplasia	0	94%	100%
Squamous cyst, total	0	1	0
Squamous cyst	0	4.2%	0
Rats with fatal tumors, total	0	8	12
Rats with fatal tumors	0	22%	26%
Mean (± SE) survival of rats with fatal tumors		403 ± 43 days	420 ± 31 days
Mean (± SE) survival of all rats	520 ± 10 days	479 ± 16 days	349 ± 24 days

[a] Number of animals in each group reflects unscheduled and end-of-study deaths.

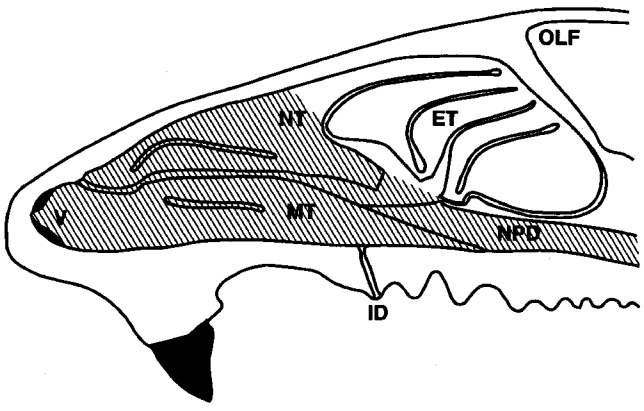

Figure 18. Distribution of lesions of the nasal mucosa in rats exposed to BDO_2. Cross-hatched area = area of lesions. V = vestibule; NT = nasoturbinate; MT = maxilloturbinate; ID = incisive duct; ET = ethmoturbinate; NPD = nasopharyngeal duct; OLF = olfactory bulb.

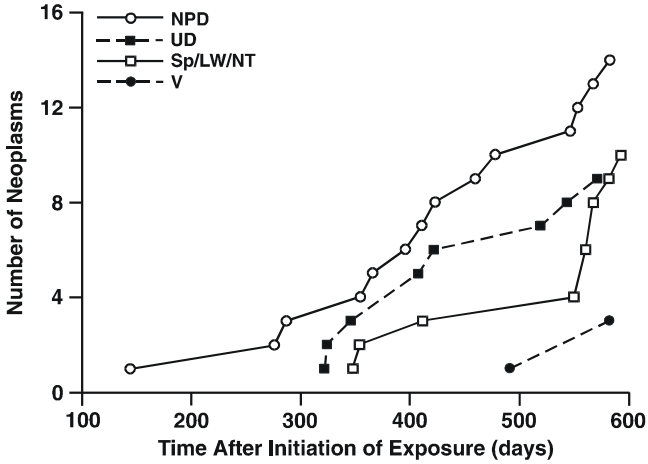

Figure 19. Cumulative incidence of neoplasms by site in the nasal mucosa of rats exposed to BDO_2. NPD = nasopharyngeal duct; UD = undetermined; Sp/LW/NT = septum, lateral wall, nasoturbinate; V = vestibule.

Table 11. Types and Locations of Neoplasms of the Nasal Mucosa in Rats Exposed to BDO_2[a]

Rat Number	Exposure (ppm)	Death Following Exposure Initiation (days)	Site[b]/Level[c]	Nasal Epithelium of Origin	Squamous Metaplasia at Site
Squamous Papilloma					
I117	5.0	561	Lateral Wall/2	Transitional	No
Carcinoma, In Situ					
H088	2.5	583 ES	NP duct/5	Respiratory	No
H102	2.5	550	Septum/2	Respiratory	Yes
I122	5.0	582 ES	Septum/2	Respiratory	Yes
I125	5.0	354	Septum/2	Respiratory	Yes
I127	5.0	491	Vestibule/1	Squamous	Yes
I142	5.0	348	Nasoturbinate/2	Respiratory	Yes
I152	5.0	568	Septum/2	Respiratory	Yes
I158	5.0	568	Septum/2	Respiratory	Yes
Squamous Cell Carcinoma, Localized					
H070	2.5	582 ES	Vestibule/1	Squamous	Yes
H075	2.5	554	NP duct/5	Respiratory	No
H090	2.5	144	NP duct/5	Respiratory	Yes
H092	2.5	412	Septum/2	Respiratory	Yes
I117	5.0	561	Septum/2	Respiratory	No
I118	5.0	582 ES	Vestibule/1	Squamous	Yes
I124	5.0	396	NP duct/5	Respiratory	No
I141	5.0	276	NP duct/5	Respiratory	Yes
I163	5.0	593 ES	Nasoturbinate/2	Respiratory	Yes
I164	5.0	355	NP duct/4,5	Respiratory	No
Squamous Cell Carcinoma, Invasive					
H064	2.5	411	NP duct/4,5	Respiratory	UD
H068	2.5	346	UD/1,2,3	UD	Yes
H082	2.5	460	NP duct/4,5	Respiratory	Yes
H093	2.5	478	NP duct/3,4,5	Respiratory	Yes
H100	2.5	324	UD/1,2,3	UD	Yes
Squamous Cell Carcinoma, Invasive (Concluded)					
I116	5.0	408	UD/1,2,3	UD	Yes
I134	5.0	520	UD/2,3,4,5	UD	No
I135	5.0	322	UD/1,2,3	UD	Yes
I136	5.0	544	UD/1,2,3	UD	Yes
I138	5.0	287	NP duct/4,5	Respiratory	Yes
I151	5.0	366	NP duct/4,5	Respiratory	No
I158	5.0	568	NP duct/4,5	Respiratory	Yes
I159	5.0	572	UD/1,2,3	UD	Yes
I162	5.0	423	NP duct/3,4,5	Respiratory	No
Adenocarcinoma					
I126	5.0	547	NP duct/5	Respiratory	No
Sarcoma					
H069	2.5	422	UD/1,2,3,4,5	UD	NA
I116	5.0	408	UD/1,2	UD	NA

[a] ES = end-of-study deaths; NP = nasopharyngeal; UD = undetermined; NA = not applicable.

[b] Anatomic site of origin within nose.

[c] Cross-sectional levels involved.

mucosa that was prominent, especially in the mucosa lining the main airflow pathway through the nose. Some of the squamous metaplasia had foci of dysplasia and marked hyperkeratosis (Figure 20). Only one squamous cyst and one squamous papilloma were seen (Figure 21). Thus, the squamous cell carcinoma seemed to arise from squamous metaplasia. All seven cases of carcinoma in situ were set in squamous metaplasia. The squamous metaplasia involved both the respiratory and transitional epithelium. However, most carcinomas arose from metaplasia involving the respiratory epithelium lining the nasal septum or the nasopharyngeal duct.

Morphologically, the squamous carcinomas ranged in malignancy from carcinoma in situ to invasive carcinoma. The carcinomas in situ were dysplastic squamous lesions that had not penetrated the basement membrane. Localized squamous cell carcinomas were small dysplastic squamous lesions that had breached the basement mem-

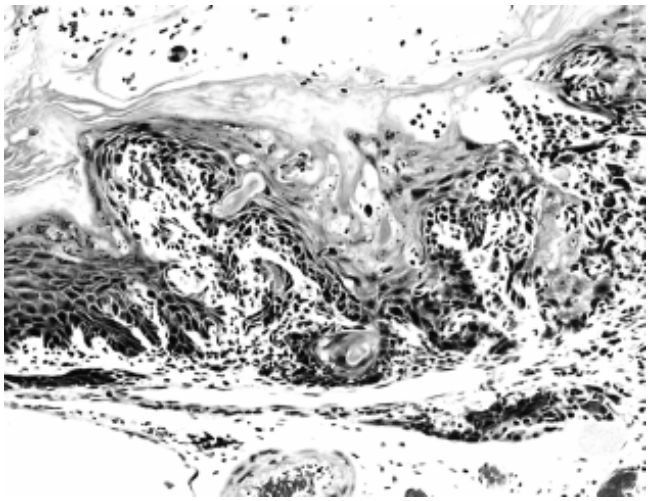

Figure 20. Photomicrograph of squamous metaplasia with dysplasia and hyperkeratosis (left) blending into squamous cell carcinoma (right) in a rat (H059) 554 days after exposure to 2.5 ppm BDO_2. Magnification × 200; hematoxylin and eosin stain.

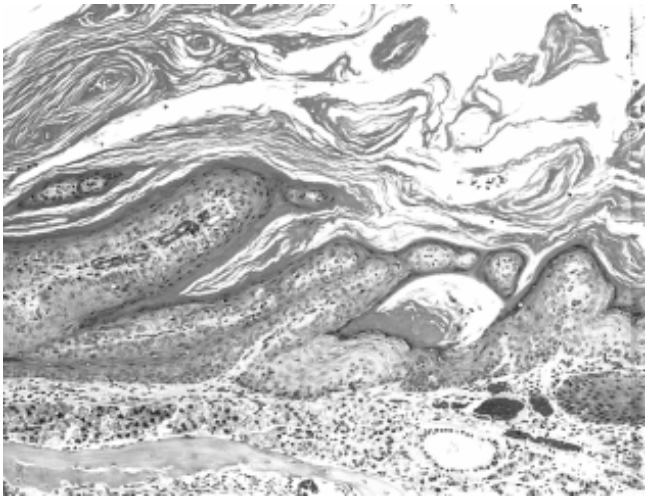

Figure 21. Photomicrograph of squamous papilloma arising from the transitional epithelium of the lateral wall of the nasal cavity in a rat (I117) 561 days after exposure to 5.0 ppm BDO_2. Magnification × 120; hematoxylin and eosin stain.

brane, invading soft tissues. Their growth was primarily along the surface of the epithelium. The invasive squamous cell carcinomas were large dysplastic lesions that had invaded bones, nerves, or vessels (Figure 22). None of the carcinomas metastasized. Several carcinomas invaded through the bones surrounding the nasal cavity into the oral cavity or subcutis on the dorsum of the nose. Only in these few cases was there gross evidence of a nasal tumor before the head was sectioned.

One neoplasm, arising from the nasopharyngeal duct, was classified as an adenocarcinoma of the respiratory epithelium (Figure 23). It was locally invasive and characterized by small nests of epithelial cells, some with mucin differentiation.

Two neoplasms were classified as sarcomas. Both were very aggressive in their growth pattern. One appeared to arise from around the root of the incisor tooth. This neoplasm metastasized to the lung. The other sarcoma was very locally invasive. Both neoplasms were composed of spindle-shaped cells that were clearly anaplastic.

Neoplasms were not increased in other organs of the body. Table 12 indicates the neoplasms noted in various organs. Gross lesions indicative of neoplasms, but seen in organs not routinely sampled, were taken for histopa-

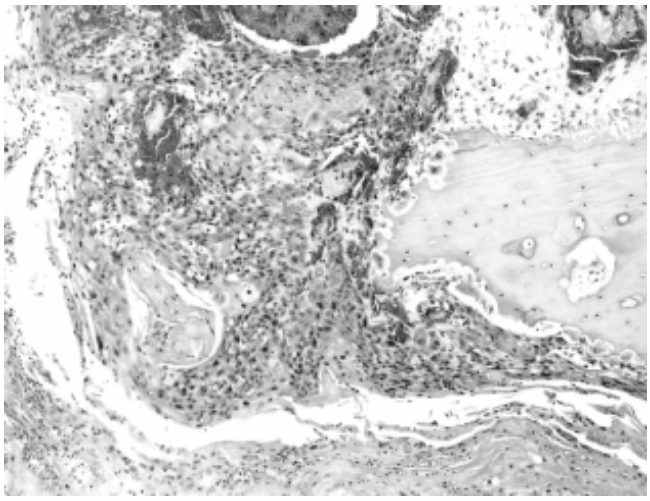

Figure 22. Photomicrograph of squamous cell carcinoma invading bone in a rat (H068) 346 days after exposure to 2.5 ppm BDO_2. Magnification × 120; hematoxylin and eosin stain.

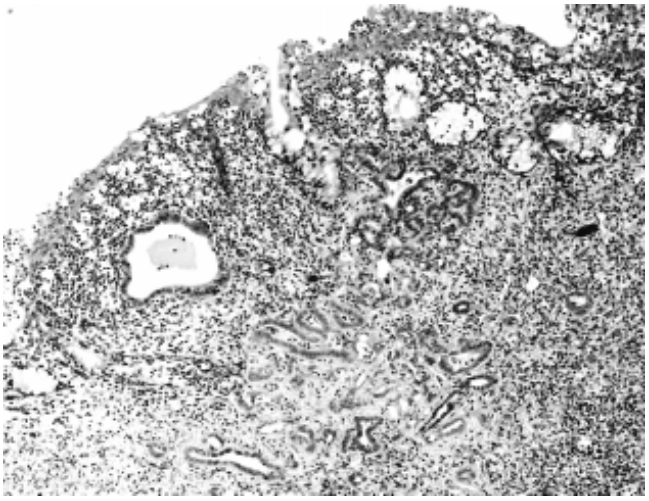

Figure 23. Photomicrograph of adenocarcinoma of the respiratory epithelium of the nasopharyngeal duct in a rat (I126) 542 days after exposure to 5.0 ppm BDO_2. Magnification × 100; hematoxylin and eosin stain.

Table 12. Neoplasms in Rats Exposed to BDO_2

Organ or Tissue[a]	Tumor Type	BDO_2 Exposure Group[b] (ppm) 0	2.5	5.0
End-of-Study Deaths				
Nasal mucosa	Benign	0/17	0/11	0/2
	Malignant	0/17	2/11	2/2
Liver	Benign	0/17	0/11	0/2
Pancreas	Benign	0/17	2/11	0/2
Stomach	Benign	0/17	0/11	0/2
Mammary gland	Benign	8/17	4/11	0/2
	Malignant	1/17	0/11	0/2
Ovary	Benign	0/17	0/11	0/2
Uterus	Benign	0/17	1/11	0/2
Unscheduled Deaths				
Nasal mucosa	Benign	0/30	0/37	1/46
	Malignant	0/30	10/37	21/46
Liver	Benign	0/31	0/37	1/46
Pancreas	Benign	0/31	0/35	0/44
Stomach	Benign	0/31	1/34	0/46
Mammary gland	Benign	16/31	8/36	7/45
	Malignant	4/31	2/36	1/45
Ovary	Benign	1/31	0/37	0/46
Uterus	Benign	0/30	1/36	0/43

[a] No neoplasms were found in protocol tissues not included here.

[b] Data are presented as [number of tumors observed]/[number of animals examined].

thology. Thus, only a few tissues were sampled for organs not on the protocol list of tissues (Table 13).

Histopathology of Mice

Nonneoplastic Lesions Few morphologic lesions were found in the four mice exposed to 5.0 ppm BDO_2 that died during the last week of exposure. A mild inflammation of the nasal mucosa was present in only one animal. Two mice had a reduced number of erythropoietic cells in the bone marrow. No conclusions could be made on the cause of death in any of these mice.

As in rats, the only exposure-related, nonneoplastic lesions found in mice at the end of exposure or later were in the nasal mucosa. These lesions were centered in the anterior portions of the nasal mucosa around the main airflow pathway through the nose (Figure 24). However, the nasopharyngeal duct was not affected. The extent and severity of the lesions varied with dose and time after exposure.

Table 13. Neoplasms in Nonprotocol Tissues[a] in Rats and Mice

Organ or Tissue	Tumor Type	BDO_2 Exposure Group[b] (ppm) 0	2.5	5.0
Rats				
Adrenal	Benign	0/3	0/7	1/4
	Malignant	0/3	1/7	0/4
Pituitary	Benign	26/28	20/21	7/8
Spleen	Benign	0/0	1/2	0/7
Mice				
Thyroid gland	Benign	0/22	1/26	0/30
Skin	Benign	0/1	1/1	0/1
	Malignant	1/1	0/1	0/1
Spleen	Benign	0/6	0/2	2/4
	Malignant	1/6	0/2	0/4
Adrenal	Benign	0/1	0/0	0/1
	Malignant	0/0	0/0	1/1
Pituitary	Benign	1/1	0/0	0/0

[a] These tissues were examined when gross lesions indicative of neoplasms were observed during necropsy. Some tissues were lost to follow-up.

[b] Data are presented as [number of tumors observed]/[number of animals examined].

In mice killed at the end of exposure to 5.0 ppm, the lesions were characterized by necrosis and inflammation of the mucosa lining the anterior nasal cavity and by minimal atrophy of the underlying turbinate bones. A fibrinopurulent exudate was present in the nasal cavity. A few mice had squamous metaplasia of the nasal mucosa and atrophy of the anterior portions of the olfactory epithelium lining the dorsal meatus. In mice exposed to 2.5 ppm, the

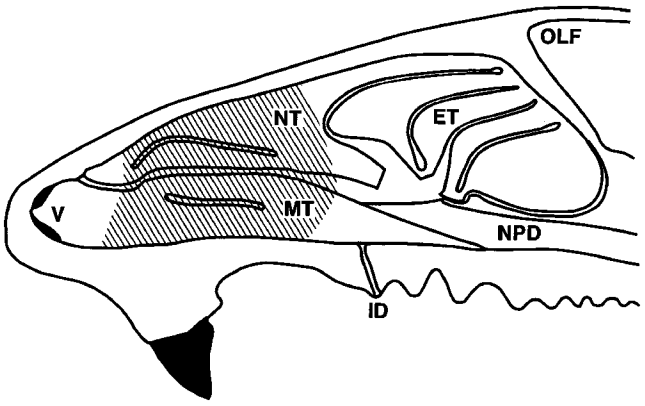

Figure 24. Distribution of lesions of the nasal mucosa in mice exposed to BDO_2. Cross-hatched area = area of lesions. V = vestibule; NT = nasoturbinate; MT = maxilloturbinate; ID = incisive duct; ET = ethmoturbinate; NPD = nasopharyngeal duct; OLF = olfactory bulb.

lesions were similar in nature but much less severe and frequent. Inflammation was prominent, but there was no atrophy of the turbinate bones.

In the mice that died or were found moribund and euthanized (only one died earlier than 510 days after start of exposure), chronic inflammation and squamous metaplasia characterized the nasal lesions. Neutrophils were a major infiltrating cell and component of exudate into the nasal lumen. In a few cases, the inflammation was severe enough to cause erosion of the nasal septum, creating a fenestration between the two halves of the nasal cavity. The maxilloturbinates and the anterior portion of the nasoturbinates were atrophied in the higher-dose animals. The lesions were more extensive and severe in the high-dose mice. In the most severe lesions, hyperkeratosis was prominent. In all cases, however, the lesions were less severe than those in rats similarly exposed.

The respiratory epithelium of the nasal mucosa not directly infiltrated with inflammatory cells was frequently degenerative. Cytoplasmic vacuolization and accumulations of protein crystals characterized these changes. The anterior portion of the olfactory epithelium in these same animals exhibited similar degenerative changes.

Fibro-osseous proliferative lesions of the bones of the skull were occasionally found in association with the inflammatory lesions of the nasal epithelium. These lesions thickened and distorted the maxilla, turbinates, frontal bones, and hard palate in some animals. The proliferations appeared secondary to the inflammation, not a direct effect of the exposure. No such lesions were seen in the skulls of unexposed animals. A similar fibro-osseous lesion was present in routine sections of femur similar to that previously described in control mice. These lesions were found with similar frequency in both exposed and control mice.

Neoplasia Neoplastic lesions in mice are listed in Table 14. In general, the mice had only one lesion; however, some animals did have multiple lesions in the same or in different organs. On the basis of individual organs or tissues, the number of observed lesions was generally too small to perform a meaningful statistical analysis. The exception to this was the Harderian gland, for which a contingency table was constructed. The chi-square test indicated a statistically significant effect associated with the high dose of BDO_2 (uncorrected $p = 0.015$; Bonferroni-corrected $p < 0.05$).

The contingency table analyses are shown in Table 15. Only animals surviving to the end-of-study deaths were included. A statistically significant increase in presence of lesions was observed in mice exposed to 5.0 ppm BDO_2 relative to mice exposed to 2.5 ppm BDO_2 (uncorrected $p = 0.007$; Bonferroni-corrected $p < 0.05$). When compared with controls, the difference was not statistically significant (uncorrected $p = 0.07$). When grouped to examine only respiratory lesions or only reproductive lesions, no statistically significant effects were observed.

Table 14. Neoplasms in Mice Exposed to BDO_2

		BDO_2 Exposure Group[b] (ppm)		
Organ or Tissue[a]	Tumor Type	0	2.5	5.0
Unscheduled Deaths				
Nasal mucosa	Malignant	0/4	0/2	0/3
Harderian gland	Benign	0/4	0/2	0/8
Ovary	Benign	0/3	0/2	0/6
Lung	Benign	0/4	0/2	0/8
Uterus	Benign	0/4	0/2	0/6
Femur	Malignant	1/4	0/2	0/7
Tissue with lymphoma	Malignant	0/4	1/2	1/8
Liver	Benign	0/4	0/2	0/7
Mammary gland	Malignant	1/3	0/2	0/8
Pancreas	Benign	0/4	0/2	0/7
End-of-Study Deaths				
Nasal mucosa	Malignant	0/40	0/42	1/36
Harderian gland	Benign	0/40	2/42	5/36
Ovary	Benign	0/40	0/41	2/36
Lung	Benign	1/40	1/42	4/36
	Malignant	2/40	1/42	0/36
Uterus	Benign	0/40	0/42	1/36
Femur	Malignant	0/40	1/42	0/36
Tissue with lymphoma	Malignant	2/40	1/42	2/36
Liver	Benign	2/40	1/42	1/36
	Malignant	1/40	1/42	0/36
Mammary gland	Malignant	0/40	0/42	0/36
Pancreas	Benign	0/40	0/42	1/36
	Malignant	1/40	0/42	0/36

[a] No neoplasms were found in protocol tissues not listed. Some tissues were lost to follow-up.

[b] Data are presented as [number of tumors observed]/[number of animals examined].

The one tumor in the nasal mucosa was a squamous cell carcinoma in situ. The neoplasm arose from a field of squamous metaplasia on the lateral wall of the nasal cavity. Transitional nasal epithelium normally lines this portion of the nasal cavity. The exuberant growth and production of keratin by the neoplasm projected into the nasal lumen

Table 15. Contingency Table Analyses of Neoplastic Lesions in Mice

BDO_2 Concentration (ppm)	Number of Animals with Lesions	
	0	1 or More
All Tumors χ^2 p = 0.021		
0	30	10
2.5	35	7
5.0	20	16
Reproductive Tumors χ^2 p = 0.121		
0	40	0
2.5	41	1
5.0	33	3
Respiratory Tumors χ^2 p = 0.378		
0	36	4
2.5	40	2
5.0	31	5

nearly occluding the left half of the nasal passage. No neoplasms were found in the nasopharyngeal duct.

The Harderian gland tumors were all adenomas. They appeared to arise de novo, not from areas of preexisting lesions as was the case in the tumor from the nasal epithelium.

Gross lesions indicative of neoplasms, but seen in organs not routinely sampled, were taken for histopathology. Thus, only a few tissues were sampled for organs not on the protocol list of tissues (Table 13).

DISCUSSION

GENERAL

The results of the study were contrary to expectations. The mice were expected to have at least as many tumors induced by inhalation exposure to BDO_2 as the rats, but that was not the case. Despite the indications from preliminary studies (Appendix A) that mice received approximately twice the dose of BDO_2 to blood and lungs as did the rats, the mice did not develop the number of nasal tumors observed in rats.

The daily dose of BDO_2 in the lungs of the exposed rats and mice can be estimated from earlier studies (Table 16). The original intent was to expose rodents to enough BDO_2 to induce lung tumors but not lymphomas that cause premature deaths in mice. The highest exposure to BD that caused tumors in mice but not lymphomas was the repeated exposure to 62.5 ppm BD (Melnick et al. 1990). According to the earlier work of Thornton-Manning and coworkers (1995a), a 4-hour exposure of mice to 62.5 ppm BD results in lung concentrations of BDO_2 equal to 114 ± 37 pmol/g tissue. The data suggest that the level of BDO_2 in lung was increasing linearly during the 4-hour exposure; if one extrapolates linearly, one can estimate that the lung levels of BDO_2 would be approximately 180 pmol/g lung after a 6-hour exposure to 62.5 ppm BD (Table 16).

Preliminary dosimetry studies (Appendix A) indicated that a 6-hour exposure to 12 ppm BDO_2 yields a level of BDO_2 in the lung of 2,900 pmol/g tissue. If, as shown in Table 16, one calculates the dose to lung (total pmol of BDO_2/g tissue) in the chronic exposure (~520 days), the estimate is 93,600 pmol of BDO_2/g tissue. This can be compared with the total dose of BDO_2 to lung in the current study of 36,250 pmol/g tissue in mice exposed for 6 weeks to BDO_2. Thus, the BDO_2-exposed mice received approximately 40% (5.0 ppm) or 20% (2.5 ppm) of the BDO_2 dose received by the lungs of mice in chronic exposures to 62.5 ppm BD. Considering that lung tumors were found in mice inhaling only 6.25 ppm BD in the chronic-exposure studies, the dose to the lung in the BDO_2-exposed mice in the current study was within the range of BDO_2 doses received by BD-exposed mice that developed lung tumors.

Table 16. Estimated Cumulative Dose of BDO_2 Delivered to Lungs of Mice

Exposure	Total BDO_2 to Lung (pmol/g tissue)
Single	
62.5 ppm BD, 6 hours[a]	180
12 ppm BDO_2, 6 hours[b]	2,900
Repeated	
62.5 ppm BD, 6 hr/day, 520 days[c]	93,600[d]
2.5 ppm BDO_2, 180 hours[e]	18,125
5.0 ppm BDO_2, 180 hours[e]	36,250[f]

[a] Thornton-Manning et al. (1995a).

[b] Henderson et al. (1999).

[c] Chronic bioassay study (Melnick et al. 1990).

[d] Estimated cumulative dose delivered to lung on basis of data from single exposures by Thornton-Manning et al. 1995a; calculated as 62.5 ppm BD = 180 pmol BDO_2/g/day × 520 days = 93,600 pmol/g.

[e] Current study.

[f] Estimated cumulative dose delivered to lung on basis of single exposures by Henderson et al. 1999; calculated for six-week exposure (180 hours) at 5.0 ppm BDO_2 = 2,900 pmol BDO_2/g/day × 5 ppm/12 ppm × 30 days = 36,250 pmol/g. Dose to lung in current study is approximately 40% (5.0 ppm) or 20% (2.5 ppm) of the cumulative dose of BDO_2 received by lungs of mice in chronic exposures to 62.5 ppm BD.

It should be noted that the above estimates are based on single exposures, and the extrapolation to repeated exposures may not be linear. Repeated exposures might induce enzymes that lead to either increases or decreases in the lung BDO_2 levels. It should also be noted that the time course of the rise in lung levels of BDO_2 in mice exposed directly to BDO_2 may be more rapid than the rise in lung levels of BDO_2 in mice exposed to BD (due to the time required to metabolize BD to BDO_2). If this is so, our calculations would underestimate the relative lung dose of BDO_2 received by the rodents exposed directly to BDO_2 compared with rodents exposed to BD. Thus, one must consider the estimates of dose in Table 16 as "ballpark" figures.

Even if the estimate of the absolute dose received by the rodents has a good deal of uncertainty associated with it, it is clear from the preliminary studies that the respiratory tract and blood of the mice had a higher concentration of BDO_2 than did those of the rats (approximately twofold). The dose received by the mice was sufficient to cause a decrease in blood lymphocytes. Yet upper respiratory tract tumors occurred to a much higher degree in the rats than the mice. One may conclude that the epithelial tissues of the upper respiratory tract of the rat are sensitive enough to the tumorigenic activity of BDO_2 that, if the metabolite were present in vivo following BD exposures, one would expect to see tumors. Thus, the minimal tumorigenic response of rats to BD inhalation is not due to lack of sensitivity to BDO_2.

The question remains as to why the mice, which are exquisitely sensitive to inhaled BD, did not develop respiratory tract tumors after exposure to BDO_2. One explanation might be that the dose was not high enough. The calculated estimates of dose suggest that it should have been sufficient, and the exposure was sufficient to cause portal-of-entry tumors in rats. A more likely explanation is that the site of formation of BDO_2 within the cell may be important for its tumorigenic action. The transport of externally administered BDO_2 into the cell to the critical sites on the DNA may allow detoxification of the molecule before it reaches the target site. Thus, the biologically effective dose may be greatly diminished by administering the compound external to the cell compared with the metabolic formation of the compound within the cell. However, the external dose received by the rat was sufficient to induce tumors in the upper respiratory tract.

Another possible reason for the fact that the BDO_2-exposed mice had only one nasal tumor is that glutathione *S*–transferase (GST) activities, which lead to detoxication of the diepoxide, are much higher in the cytosol of mouse tissues than in rat tissues (Boogaard et al. 1996). The externally administered diepoxide would have to pass through the cytosol containing the GST before reaching the target DNA molecules in the nucleus. The GST activity in the cytosol would be expected to detoxicate the diepoxide to a greater extent in mice than in rats, which would lead to a greater tumor response in rats.

One aspect of the exposure that must be considered is the stereochemical form of the diepoxide used in the exposures versus the form of the diepoxide produced by metabolism of BD in vivo. Studies by Nieusma and coworkers (1997) using hepatic microsomes indicate that mouse microsomes produce slightly more (*S*)-BDO than (*R*)-BDO and produce significantly more BDO_2 from (*S*)-BDO than from (*R*)-BDO. (*R*)-BDO was found to be more toxic toward rat hepatocytes than was (*S*)-BDO, and *meso*-BDO_2 was the most toxic BDO_2 enantiomer. The same investigators (Nieusma et al. 1998) reported that (*R*)-BDO depletes cytosolic reduced glutathione (GSH) in isolated rat hepatocytes faster than does (*S*)-BDO and that (*S*,*S*)- and *meso*-BDO_2 depleted cytosolic GSH faster than the (*R*,*R*)-BDO_2. Krause and Elfarra (1997) reported that human cytochrome P450 2E1 preferentially forms *meso*-BDO_2 from BDO and that the *meso*-BDO_2 was preferentially hydrolyzed by human liver microsomes. If the *meso*-BDO_2 is also preferentially formed in mice and the *meso* form is one of the more toxic enantiomers, then the exposure to (±)-BDO_2 in this study may have been less effective as a tumorigen than the BDO_2 formed in vivo.

The mice were not totally insensitive to BDO_2-induced neoplasia because contingency table analyses (Table 15) indicated a statistically significant increase in neoplasia (all organs) in mice exposed to 5.0 ppm BDO_2 compared with mice exposed to 2.5 ppm BDO_2. This trend, however, is driven by the Harderian gland tumors.

K-*ras* MUTATIONS

The only tumors observed in the respiratory tract were unfortunately in the nose, which underwent a decalcification process along with fixation. The decalcification process used to fix these tissues apparently denatured the DNA to the extent that fragments could not be amplified. Thus, this specific aim of the study was not accomplished.

HEMATOLOGY

In rats at the end of 6 weeks of exposure, there were no differences in the hematological parameters of the control and exposed animals. At the end-of-study deaths, the red blood cell numbers in the few remaining high-dose rats were elevated. The significance of this increase is unknown.

At the end of the 6-week exposure, the mice were in poor clinical condition, and four had died. Ulcerative

rhinitis was present, and the animals showed signs of stress. The decreased lymphocyte counts and increased neutrophil counts reflected the clinical picture. At the end-of-study deaths, similar but less severe hematological changes were present. At neither time of scheduled death was there any evidence of anemia as reported for NIH Swiss mice exposed repeatedly to BD for 6 weeks (Irons et al. 1986).

HISTOPATHOLOGY

Rats

The site specificity of nasal lesions has been noted (Morgan and Monticello 1990; Morgan 1997). Both nonneoplastic and neoplastic responses in the nasal passages generally occur in specific locations. Two major factors influence the distribution of lesions induced by inhaled chemicals: regional deposition of the chemical and tissue susceptibility. Air-flow patterns and solubility are important factors in the regional deposition. Tissue susceptibility is influenced by the different types of epithelia in the nasal mucosa, each with characteristic morphologic, biochemical, and physiologic properties. Regional metabolism is high in the olfactory epithelium, resulting in potential metabolism of inhaled chemicals to toxic metabolic intermediates or to less toxic compounds.

The neoplastic and nonneoplastic lesions induced in rats by BDO_2 were distributed throughout the nasal mucosa, from the vestibule in the anterior portion of the nasal cavity to the nasopharyngeal duct in the posterior portion. The only spared portion of the nasal mucosa was the olfactory epithelium. This sparing of the olfactory epithelium is not unexpected since no metabolism is thought to be needed for BDO_2 to be toxic or carcinogenic, and the high enzymatic activity of the olfactory tissue may have hydrolyzed the BDO_2. This distribution of lesions covers a much greater area than that observed after exposure to other nasal toxicants or carcinogens and may be related to the high exposure concentration. A lower concentration may give a more restricted distribution pattern of lesions (Morgan 1997). On the other hand, the distribution of BDO_2 lesions may result from characteristics related to its chemical nature. For example, exposure of rats to formaldehyde results in inflammatory lesions and carcinomas of the respiratory epithelium lining the lateral meatus and nasal septum in the anterior nasal passages (Morgan et al. 1986). Exposure of rats to ozone results in inflammatory lesions of the transitional epithelium lining the lateral wall of the anterior nasal passages (Johnson et al. 1990). Both of these compounds probably act as direct genotoxic agents or nasal irritants. The location of the lesions may relate to the relatively soluble nature of these agents with most of the dose being delivered to the anterior portion of the nasal cavity.

The wide distribution of lesions in the nasal mucosa after inhalation of BDO_2 is somewhat similar to that of nasal lesions in rats after the inhalation of alkylating agents (Sellakumar et al. 1987). Like BDO_2, alkylating agents are direct-acting carcinogens. A study of five alkylating agents with differing hydrolysis rates showed that two agents, beta propriolactone and methylmethane sulfonate, produce high rates of nasal neoplasms. The lesions and neoplasms were site specific, involving primarily the respiratory and transitional epithelium of the anterior portion of the nasal cavity. As with BDO_2-exposed rats, no direct compound-related lesions were seen in the olfactory epithelium of the posterior region of the nasal cavity. The involved epithelium showed necrosis, ulceration, and acute inflammation. In some cases, there were squamous metaplastic and dysplastic lesions, leading to carcinogenesis. The carcinomas were primarily of the squamous type, involving the nasomaxillary turbinates, septum, and lateral walls. Even with these potent carcinogens, however, lesions and neoplasms were not seen in the nasopharyngeal duct.

One factor that may be important in the induction of nasal neoplasms in rats exposed to BDO_2 was the continued presence of inflammation in the nasal mucosa throughout the entire 18-month observation period. Although there was only one scheduled death at the end of exposure, interim deaths showed that inflammation was more intense 9 months after exposure than at the end of exposure. From 9 to 18 months after the end of exposure, the intensity of the inflammation was relatively constant. What maintained the inflammation after the cessation of exposure is unknown. Neoplasms of the respiratory tract are known to arise in direct association with chronic injury, as is the case with formaldehyde (Feron and Woutersen 1989).

Mice

In the current study, mice exposed to BDO_2 for 6 weeks and observed for 18 months longer did not have the same neoplastic response as $B6C3F_1$ mice exposed to BD at 625 ppm or 1,250 ppm for 104 weeks or at 6.25 to 625 ppm for 2 years (Miller et al. 1989; Miller and Boorman 1990). In mice exposed chronically to BD, there were significant increases of malignant lymphomas, cardiac hemangiosarcomas, alveolar-bronchiolar neoplasms, and squamous neoplasms of the forestomach in males and females, and adenocarcinomas of the mammary gland, granulosa cell tumors of the ovary, and heptocellular tumors in female mice. Brain tumors and preputial carcinomas in male mice and Zymbal's gland carcinomas in both sexes of mice were seen at low incidences, but because

of their rare occurrence in untreated mice, they may have been related to treatment. No neoplasm or other lesion was described in the nasal mucosa. Some of the neoplasms in BD-exposed mice in other studies were seen relatively soon after the initiation of exposure. The malignant lymphomas were observed at 20 weeks of exposure in one study (Huff et al. 1985) and at 23 weeks in the other (Melnick et al. 1990). Cardiac hemangiosarcomas were observed at 32 weeks of exposure in one study (Huff et al. 1985) and at 41 weeks in the other study (Melnick et al. 1990). Although the time to first observation of a neoplasm may well relate to the dose of the chemical, these observations show that these two tumor types may be observed well before the end of life span.

Proliferative lesions were seen in some organs associated with subsequent observation of neoplasms in mice exposed to BD (Miller and Boorman 1990). For example, endothelial hyperplasia in the myocardium of the ventricles was a lesion associated with the development of cardiac hemangiosarcoma. Epithelial hyperplasias were associated with an increase of neoplasms in the lung and forestomach. Such proliferations in the heart, lung, and forestomach were not seen in any of the mice exposed to BDO_2 in this study.

BDO_2 induced a much higher prevalence of nasal tumors in rats than in mice. Rats are known to be more susceptible to the induction of epithelial tumors in the nasal cavity than are mice (Brown et al. 1991). The induction of squamous cell carcinomas in the nasal cavity of rats by another irritant, formaldehyde, has been well described (Swenberg et al. 1980). As in the current study, when both rats and mice were exposed to formaldehyde for 24 months, there was squamous metaplasia in the nasal cavities of both rats and mice, but squamous cell carcinomas developed almost exclusively in the rats (Kerns et al. 1983).

In contrast to the current study, nonneoplastic lesions in the nasal cavity of both the rats and the mice regressed after the exposures to formaldehyde ended. Regression of nasal lesions after stopping exposure to an irritant was also observed in rats exposed to tobacco smoke (Maples et al. 1993). In the current study, however, the BDO_2-induced nasal lesions regressed in the mouse but not in the rat. In fact, the nasal lesions in the rat worsened with time following exposure. The basis for this difference in progression of nasal lesions from metaplasia to neoplasia in the two species could be the subject of an important follow-up study.

The squamous tumors arose from squamous metaplasia in the nasal mucosa of rats in the current study. Stages leading from well-differentiated squamous metaplasia to dysplasia to anaplasia could be seen in tissue sections. Chronic inflammation was invariably associated with the metaplastic lesions. It is tempting to speculate that the initial exposure to BDO_2 resulted in genetic changes in the exposed epithelium with subsequent inflammation and that the associated increased cell proliferation promoted the initiated cells. This scenario is similar to that proposed for the induction of nasal tumors by formaldehyde (Monticello and Morgan 1994).

An apparently essential factor in the progression to neoplasia is the persistence of inflammation. Few other inhalation studies have involved observations for regression of lesions. However, several studies have shown a regression of inflammation and squamous metaplasia in the nasal mucosa within weeks after cessation of inhalation exposure to formaldehyde (Kerns et al. 1993) or cigarette smoke (Maples et al. 1993). One study in rats (Tesfaigzi et al. 1996) examined the role of a small proline-rich protein (SPR1) in induction and regression of squamous metaplasia after inhalation of cigarette smoke. During the transition of the nasal mucociliary epithelium to squamous metaplasia, the amount of SPR1 messenger RNA (as detected by in situ hybridization with a complementary RNA probe) increased in the epithelial and mesenchymal cells. Conversely, the SPR1 messenger RNA decreased when the metaplasia regressed, indicating that the SPR1 protein could have a role in cellular differentiation in squamous metaplasia. Other studies of SPR1 expression have shown that regions of squamous tumors in close proximity to foci of inflammatory cells show strong immunostaining for SPR1, suggesting that SPR1 expression is induced by cytokines or growth factors secreted from inflammatory cells (Yaar et al. 1995). These findings are evidence that inflammation does play a role in squamous metaplasia. Results of the current study present an intriguing question: How is inflammation in the nasal mucosa of rats maintained after the cessation of the 6-week exposure to BDO_2? This question will be the subject of future research.

In summary, the results of this study indicate that BDO_2, a metabolite of BD, is carcinogenic in the upper respiratory tract of exposed rats. An increase in respiratory tract tumors was not observed in similarly exposed mice despite the fact that preliminary studies indicated mice should have received twice the dose to tissue compared with rats. Detoxification of BDO_2 by the GST activity in cytosol, which is higher in mouse cells than rat cells, may explain, in part, the species differences in response to inhaled BDO_2. The results indicate the importance of the formation site of BDO_2 in the expression of its carcinogenicity.

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REFERENCES

Bechtold WE, Strunk MR, Thornton-Manning JR, Henderson RF. 1995. Analysis of butadiene, butadiene monoxide, and butadiene dioxide in blood by gas chromatography/gas chromatography/mass spectroscopy. Chem Res Toxicol 8:182–187.

Bond JA, Dahl AR, Henderson RF, Dutcher JS, Mauderly JL, Birnbaum LS. 1986. Species differences in the disposition of inhaled butadiene. Toxicol Appl Pharmacol 84:617–627.

Boogaard PJ, Bond JA. 1996. The role of hydrolysis in the detoxification of 1,2:3,4-diepoxybutane by human, rat, and mouse liver and lung in vitro. Toxicol Appl Pharmacol 141(2):617–627.

Boogaard PJ, Sumner SCJ, Bond JA. 1996. Glutathione conjugation of 1,2:3,4-diepoxybutane in human liver and rat and mouse liver and lung in vitro. Toxicol Appl Pharmacol 136:307–316.

Brown HR, Monticello TM, Maronpot RR, Randall HW, Hotchkiss JA, Morgan KT. 1991. Proliferative and neoplastic lesions in the rodent nasal cavity. Toxicol Pathol 19:358–372.

Charles River Laboratories. 1992. http://www.criver.com/techdocs/lesions/lesions.html.

Csanády GA, Guengerich FP, Bond JA. 1992. Comparison of the biotransformation of 1,3-butadiene and its metabolite, butadiene monoepoxide, by hepatic and pulmonary tissues from humans, rats and mice. Carcinogenesis 13:1143–1153.

Dahl AR, Sun JD, Birnbaum LS, Bond JA, Griffith WC, Mauderly JL, Muggenburg BA, Sabourin PJ, Henderson RF. 1991. Toxicokinetics of inhaled 1,3-butadiene in monkeys: Comparison to toxicokinetics in rats and mice. Toxicol Appl Pharmacol 110:9–19.

Feron VJ, Woutersen RA. 1989. Role of tissue damage in nasal carcinogenesis. In: Nasal Carcinogenesis in Rodents: Relevance to Human Health Risk (Feron VJ, Bosland MC, eds). pp. 76-84. Pudoc, Wageningen, Veldhoven, The Netherlands.

Gervasi PG, Citti L, Del Monte M, Longo V, Benetti D. 1985. Mutagenicity and chemical reactivity of epoxidic intermediates of the isoprene metabolism and other structurally related compounds. Mutat Res 156:77–82.

Goodrow T, Reynolds S, Maronpot R, Anderson M. 1990. Activation of K-*ras* by codon 13 mutations in C57BL/6 C3H F_1 mouse tumors induced by exposure to 1,3-butadiene. Cancer Res 50(4):4818–4823.

Henderson RF, Hahn FF, Benson JM, Barr EB, Bechtold WE, Burt DG, Dahl AR. 1999. Dosimetry and acute toxicity of inhaled butadiene diepoxide in rats and mice. Toxicol Sci 51:146–152.

Himmelstein MW, Turner MJ, Asgharian B, Bond JA. 1994. Comparison of blood concentrations of 1,3-butadiene and butadiene epoxides in mice and rats exposed to 1,3-butadiene by inhalation. Carcinogenesis 15:1479–1486.

Himmelstein MW, Asgharian B, Bond JA. 1995. High concentrations of butadiene epoxides in livers and lungs of mice compared to rats exposed to 1,3-butadiene. Toxicol Appl Pharmacol 132:281–288.

Huff JE, Melnick RL, Solleveld HA, Haseman JK, Powers M, Miller RA. 1985. Multiple organ carcinogenicity of 1,3-butadiene in B6C3F_1 mice after 60 weeks of inhalation exposure. Science 277:548–549.

Irons RD, Smith CN, Stillman WS, Shah RS, Steinhagen WH, Leiderman LJ. 1986. Macrocytic-megaloblastic anemia in male NIH Swiss mice following repeated exposure to 1,3-butadiene. Toxicol Appl Pharmacol 85:450–455.

Johnson NF, Hotchkiss JA, Harkema JR, Henderson RF. 1990. Proliferative responses of rat nasal epithelia to ozone. Toxicol Appl Pharmacol 103:143–155.

Kerns WD, Pavkov KL, Donofrio DJ, Gralla EJ, Swenberg JA. 1983. Carcinogenicity of formaldehyde in rats and mice after long-term inhalation exposure. Cancer Res 43:4382–4392.

Krause RJ, Elfarra AA. 1997. Oxidation of butadiene monoxide to meso- and (±)-diepoxybutane by cDNA-expressed human cytochrome P450s and by mouse, rat and human liver microsomes: Evidence for preferential hydration of meso-diepoxybutane in rat and human liver microsomes. Arch Biochem Biophys 337:176–184.

Maples KR, Nikula KJ, Chen BT, Finch GL, Griffith WC Jr, Harkema JR. 1993. Effects of cigarette smoke on the glutathione status of the upper and lower respiratory tract of rats. Inhalation Toxicol 5:389–401.

Melnick RL, Huff J, Chou BJ, Miller RA. 1990. Carcinogenicity of 1,3-butadiene in C57BL/6 X C3H F_1 mice at low exposure concentrations. Cancer Res 50:6592–6599.

Mery S, Gross DA, Joyner DR, Godo M, Morgan KT. 1994. Nasal diagrams: A tool for recording the distribution of nasal lesions in rats and mice. Toxicol Pathol 22(4):353–372.

Miller RA, Boorman GA. 1990. Morphology of neoplastic lesions induced by 1,3-butadiene in B6C3F_1 mice. Environ Health Perspect 86:37–48.

Miller RA, Melnick RL, Boorman GA. 1989. Neoplastic lesions induced by 1,3-butadiene in B6C3F_1 mice. Exp Pathol 37:136–146.

Monticello TM, Morgan KT. 1994. Cell proliferation and formaldehyde-induced respiratory carcinogenesis. Risk Anal 14:313–319.

Morgan KT. 1997. A brief review of formaldehyde carcinogenesis in relation to rat nasal pathology and human health risk assessment. Toxicol Pathol 25:291–307.

Morgan KT, Monticello TM. 1990. Airflow, gas deposition, and lesion distribution in the nasal passages. Environ Health Perspect 85:209–218.

Morgan KT, Jiang XZ, Starr TB, Kerns WD. 1986. More precise localization of nasal tumors associated with chronic exposure of F-344 rats to formaldehyde gas. Toxicol Appl Pharmacol 82:264–271.

Nickell-Brady C, Hahn FF, Finch GL, Belinsky SA. 1994. Analysis of K-*ras*, p53 and c-raf-1 mutations in beryllium-induced rat lung tumors. Carcinogenesis 15:257–262.

Nieusma JL, Claffey DJ, Maniglier-Poulet C, Imiolczyk T, Ross D, Ruth JA. 1997. Stereochemical aspects of 1,3-butadiene metabolism and toxicity in rat and mouse liver microsomes and freshly isolated rat hepatocytes. Chem Res Toxicol 10:450–456.

Nieusma JL, Claffey DJ, Ruth JA, Ross D. 1998. Stereochemical aspects of the conjugation of epoxide metabolites of butadiene with glutathione in rat liver cytosol and freshly isolated rat hepatocytes. Toxicol Sci 43:102–109.

Owen PE, Glaister JR, Gaunt IF, Pullinger DH. 1987. Inhalation toxicity studies with 1,3-butadiene: 3. Two year toxicity/carcinogenicity study in rats. Am Ind Hyg Assoc J 48:407–413.

Owen PE, Glaister JR. 1990. Inhalation toxicity and carcinogenicity of 1,3-butadiene in Sprague-Dawley rats. Environ Health Perspect 86:19–25.

Schmidt U, Loeser E. 1985. Species differences in the formation of butadiene monoxide from 1,3-butadiene. Arch Toxicol 57:222–225.

Sellakumar AR, Snyder CA, Albert RE. 1987. Inhalation carcinogenesis of various alkylating agents. JNCI 79:285–289.

Swenberg JA, Kerns WD, Mitchell RI, Gralla EJ, Pavkov KL. 1980. Induction of squamous cell carcinomas of the rat nasal cavity by inhalation exposure to formaldehyde vapor. Cancer Res 40:3398–3402.

Tesfaigzi J, Th'ng J, Hotchkiss JA, Harkema JR, Wright PS. 1996. A small proline-rich protein, SPPR1, is upregulated early during tobacco smoke-induced squamous metaplasia in rat nasal epithelia. Am J Respir Cell Mol Biol 14:478–486.

Thornton-Manning JR, Dahl AR, Bechtold WE, Griffith WC Jr, Henderson RF. 1995a. Disposition of butadiene monoepoxide and butadiene diepoxide in various tissues of rats and mice following a low-level inhalation exposure to 1,3-butadiene. Carcinogenesis 16:1723–1731.

Thornton-Manning JR, Dahl AR, Bechtold WE, Griffith WC Jr, Pei L, Henderson RF. 1995b. Gender differences in the metabolism of 1,3-butadiene in Sprague-Dawley rats following a low-level inhalation exposure. Carcinogenesis 16:2875–2878.

Vangala RR, Laib RJ, Bolt HM. 1993. Evaluation of DNA damage by alkaline elution technique after inhalation exposure of rats and mice to 1,3-butadiene. Arch Toxicol 67:34–38.

Yaar M, Eller MS, Bhawan J, Harkness DD, DiBenedetto PJ, Gilchrest BA. 1995. In vivo and in vitro SPRR1 gene expression in normal and malignant keratinocytes. Exp Cell Res 217(2):217–226.

APPENDIX A. Preliminary Studies* on Dosimetry of 1,2,3,4-Diepoxybutane Administered by Various Routes

BACKGROUND

On October 21, 1994, a workshop was held for all investigators who were to be funded by HEI to study issues related to the toxicity of 1,3-butadiene. At that workshop there was a discussion of the route of delivery of BDO_2 in our study. The route of delivery proposed was repeated intratracheal (i.t.) instillation directly into the lung. A rep-

* The preliminary research described in this Appendix was funded by the Chemical Manufacturers Association and has been published in part elsewhere (Henderson et al. 1999).

resentative of the Chemical Manufacturers Association (CMA), Dr. Larry Griffis, suggested that exposure by inhalation might avoid the acute toxicity associated with a bolus dose and avoid the criticism that any resulting tumors might have been produced by epigenetic mechanisms. The possibility of using Alzet pumps was also to be explored. The objective of the preliminary studies was to determine the dose of BDO_2 delivered to the blood and lung by the various routes.

DOSIMETRY STUDIES

Intratracheal Instillation and Intraperitoneal Injection

For the preliminary study, the experiment was designed to dose rats and mice with amounts of BDO_2 close to the maximum tolerated dose and to determine the concentration of BDO_2 in the lung and blood at various times after the exposure. Data from the laboratory of Dr. Glenn Sipes of the University of Arizona (personal communication) indicated that repeated (30 daily) doses of 26 mg/kg were tolerated by $B6C3F_1$ mice and Sprague-Dawley rats with a weight loss of greater than 10%. Therefore, the rodents were dosed with 26 mg BDO_2/kg (i.t. or i.p.) and the amount of BDO_2 in the blood and lung determined at 5 minutes (i.t. only), 30 minutes, and 2 hours. Results are shown in Table A.1.

BDO_2 was cleared rapidly from the blood and lungs in both rats and mice, with no difference between species in clearance rates. As expected, the i.p. administration resulted in a lower dose to the lung than did the i.t. administration of the same amount of BDO_2, presumably due to first-pass metabolism in the liver following the i.p. injections.

Inhalation

In this study, rats and mice were exposed to 12 ppm BDO_2 for 6 hours, and the concentrations of BDO_2 in the lung and blood were measured at the end of the exposure. Results are shown in Table A.2. As would be expected on the basis of the difference in minute volume per kilogram of body weight in the mouse versus the rat, the mouse lungs and blood contained two to three times the concentration of BDO_2 of the rat lungs and blood. Values in the rat lung after inhalation were similar to 30-minute values in rats following i.t. administration of 26 mg/kg. The concentration of BDO_2 in the lungs of mice exposed to 12 ppm BDO_2 for 6 hours was approximately 16 times that in the lungs of mice exposed for 6 hours to 62.5 ppm BD.

Table A.1. BDO_2 Levels in Mouse and Rat Tissue After Intratracheal or Intraperitoneal Exposure[a]

		BDO_2[b] (pmol/g tissue)	
Tissue	Time (min)	Intratracheal	Intraperitoneal
Rats			
Blood	0[c]	300,000	300,000
	5	65,980 ± 1,690	NA[d]
	30	1,054 ± 470	364 ± 109
	120	57.5 ± 32.8	35.2 ± 6.0
Lung	0[c]	300,000	300,000
	5	71,200 ± 3,960	NA
	30	1,510 ± 393	265 ± 48
	120	27.2 ± 17.5	3.0 ± 0.3
Mice			
Blood	0[c]	300,000	300,000
	5	67,120 ± 9,260	NA
	30	1,339 ± 537	277 ± 47
	120	65.5 ± 38.8	2.7 ± 1.4
Lung	0[c]	300,000	300,000
	5	57,120 ± 5,090	NA
	30	7,700 ± 2,700	195 ± 66
	120	70.1 ± 21.0	14.9 ± 3.3

[a] Exposure was 26 mg BDO_2/kg body weight; n = 3 to 5 animals of each species.

[b] Values are expressed as means ± SE.

[c] The theoretical amount present immediately after dosing, if one assumes immediate and equal distribution throughout the body.

[d] NA = Not analyzed.

Table A.2. BDO_2 Levels in Mouse and Rat Tissue After a 6-Hour Inhalation Exposure to 12 ppm BDO_2[a]

	BDO_2 (pmol/g tissue)	
Tissue	Rat	Mouse
Blood	429 ± 122	1,020 ± 29
Lung	957 ± 173	2,880 ± 550

[a] Values are expressed as means ± SE; n = 3 to 5 animals of each species.

Alzet Pump

To determine the stability of BDO_2 in an Alzet pump, we assayed for BDO_2 remaining in a saline solution of BDO_2 held at 37°C for several days. The half-life of the compound was approximately 60 hours, in good agreement with earlier data reported by Gervasi and associates (1985).

It was decided not to pursue the use of the Alzet delivery system further because multiple surgeries would be necessary on animals to replace the pumps in order to maintain the dose of BDO_2 over several weeks.

HISTOPATHOLOGIC STUDIES

After the dosimetry studies, exposure by inhalation was favored because (1) inhalation allows a steady delivery of the BDO_2 to the rodents for 6 hours/day, 5 days/week; (2) the BDO_2 gets past the nose in sufficient amounts to provide large doses of BDO_2 directly to the lung; and (3) the inhalation route is feasible and practical. The following histopathology study was conducted to help determine the appropriate exposure concentrations for our study; we wanted to deliver as much BDO_2 to the lung as possible without causing acute injury that would confound the interpretation of study results.

Rats and mice were exposed for 6 hours to 18 ppm BDO_2. An exposure design under consideration for the major repeat-dosing study was to include exposures to 10 or 20 ppm BDO_2 (see section of Experimental Design below). Therefore, rodents were exposed to a concentration near the maximum exposure to be used in the repeated study to see whether acute toxicity would be induced.

The respiratory tracts of the exposed animals were examined immediately after exposure and at 1, 3, and 7 days after the exposure. Control animals exposed to air under the same conditions were examined at 3 days after the sham exposure. In addition to the respiratory tract, liver, kidney, bone marrow, heart, and ovary were examined for histopathology. Bronchoalveolar lavage fluid was analyzed for signs of an inflammatory response immediately after exposure and at 1 and 3 days after exposure.

Results are summarized in Table A.3 (histopathology) and Table A.4 (lavage fluid). Lesions in the lungs of rats and mice were minimal and were characterized by minimal increases in numbers of alveolar macrophages and slight interstitial infiltration of granulocytes. The reaction in rats was slightly more severe than in the mice.

The necrosis of the olfactory epithelium was focal, concentrated at the anterior dorsal portion of the epithelium. The lesion had begun to repair 7 days after exposure. The results indicate little tissue reaction to the BDO_2 except in the olfactory epithelium of the nose. No lesions were found in liver, kidney, marrow, heart, or ovary.

The nose is a logical target organ for the inhaled BDO_2 because the compound, which is water soluble and highly reactive, should deposit heavily in the nose. The rat olfactory epithelium was particularly sensitive to the compound. Despite the fact that the rats had approximately half the concentration of BDO_2 in blood and lungs as did the mice, the rats had a slightly greater tissue response to the compound. (The concentration of BDO_2 in the nose of the two species was not measured and may not necessarily reflect the blood concentrations.) It is interesting that the nasal olfactory epithelium was damaged by the BDO_2, but the respiratory epithelium was not. The usual interpretation of such a response is that the olfactory tissue is more sensitive because of its high metabolic activity; however, the diepoxide is already metabolized, suggesting that the olfactory tissue has some inherent sensitivity to the compound.

Bronchoalveolar lavage fluid analysis indicated little response to the inhaled BDO_2. One rat (A-008) had an influx of neutrophils and a doubling of macrophages at 1 day after the exposure. No other rodents had any response detectable in the lavage fluid.

INFLUENCE ON EXPERIMENTAL DESIGN FOR THE REPEATED-EXPOSURE STUDY

Route of Delivery

Based on the results of our preliminary studies, we decided to use the inhalation route for the repeated delivery of the BDO_2 to the lung for reasons given above.

Exposure Concentrations

As stated in our original proposal, we exposed the rodents to enough BDO_2 to induce tumors but not lymphomas that cause premature deaths in the mice. The highest exposure to BD that caused tumors in mice but not lymphomas was the repeated exposure to 62.5 ppm BD. Work by Dr. Janice Thornton-Manning at the Inhalation Toxology Research Institute (ITRI) indicates that lung concentrations of BDO_2 following a 6-hour exposure of mice to 62.5 ppm BD is approximately 180 pmol/g lung tissue. Our recent results indicate that mice and rats exposed to 12 ppm BDO_2 for 6 hours will have 2,900 (mice) and 1,000 (rats) pmol/g lung tissue at the end of exposure. The number of daily exposures to BD in the 104-week study was 520, resulting in a 94 pmol-day/g lung total exposure to BDO_2 (520 days × 180 pmol/g). In a 5-days/week, 4-week exposure to 12 ppm BDO_2, the number of exposure days would be 20, resulting in a 58 µmol-day/g lung total exposure to BDO_2 (20 × 2.9 µmol/g). By linear extrapolation, a 4-week exposure to 20 ppm BDO_2 would result in a dose of BDO_2 to the lung of 97 µmol-day/g, a total dose similar to that received by mice in the 2-year study. (It should be recognized that these are crude extrapolations based on concentrations of BDO_2 in tissues at the end of the exposure, not on area under the curve measurements. They are, however, useful to determine whether we are dealing with approximately similar doses.)

The lungs of rats and mice were not greatly affected by one 6-hour exposure to 18 ppm BDO_2, but the nasal tissues, which presumably received a higher dose, had focal necrosis of the olfactory epithelium. Exposures (6-hour) to 20 ppm BDO_2 result in a lung burden in mice equivalent to that received by 6-hour exposures to 1,700 ppm BD. The National Toxicology Program (NTP) conducted 15-day exposures of mice to BD up to 8,000 ppm, and no deaths occurred. Mice exposed for 15 days to 2,500 ppm BD even had some weight gain, although 6% less than controls. Thus, exposure to 20 ppm BDO_2, 6 hours/day, 5 days/week for 4 weeks is not likely to cause deaths.

We used 20 ppm as our higher exposure level and 10 ppm as our lower exposure level. The higher exposure ensured that the mice received a dose comparable with that received in the 2-year study. Although the rats received only about half of that dose, we did not want to use a higher dose because of the nasal necrosis observed in 1-day exposures to

Table A.3. Histopathologic Studies of BDO_2 Toxicity for Rats and Mice Exposed for 6 Hours to 18 ppm BDO_2[a]

Animal Number	Treatment	Days After Exposure	Lung			Nose
			Airway	Alveoli	Interstitium	
Rats						
A-001	Control	3	NR	AM hyperplasia (1+)	Focal infiltrate	NR
A-003	Control	3	NR	AM hyperplasia (1+)	Focal infiltrate	NR
A-004	Control	3	NR	NR	Diffused infiltrate	NR
A-005	Exposed	0	NR	AM hyperplasia (1+)	NR	NR
A-006	Exposed	0	NR	AM hyperplasia (1+)	NR	NR
A-007	Exposed	0	NR	AM hyperplasia (1+)	NR	NR
A-008	Exposed	1	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (2+)
A-010	Exposed	1	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (2+)
A-011	Exposed	1	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (2+)
A-012	Exposed	3	NR	NR	NR	Degeneration, regeneration (1+)
A-013	Exposed	3	NR	NR	NR	Degeneration, regeneration (1+)
A-014	Exposed	3	NR	AM hyperplasia (1+)	Diffused infiltrate	Degeneration, regeneration (1+)
A-015	Exposed	7	NR	AM hyperplasia (1+)	NR	Degeneration, regeneration (1+)
A-017	Exposed	7	NR	AM hyperplasia (1+)	NR	Degeneration, regeneration (1+)
Mice						
B-003	Control	3	NR	NR	NR	NR
B-004	Control	3	NR	NR	NR	NR
B-005	Exposed	0	NR	NR	NR	NR
B-006	Exposed	0	NR	NR	NR	NR
B-007	Exposed	0	NR	NR	NR	NR
B-008	Exposed	0	NR	NR	NR	NR
B-009	Exposed	1	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (2+)
B-010	Exposed	1	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (2+)
B-011	Exposed	1	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (2+)
B-012	Exposed	3	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (1+)
B-013	Exposed	3	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (2+)
B-014	Exposed	3	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (1+)
B-015	Exposed	3	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (1+)
B-016	Exposed	7	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (1+)
B-017	Exposed	7	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (1+)
B-019	Exposed	7	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (1+)
B-020	Exposed	7	NR	AM hyperplasia (1+)	NR	Focal olfactory epithelial necrosis (1+)

[a] Responses in liver, kidney, ovaries, bone marrow, and heart were not remarkable. NR = not remarkable; AM = alveolar macrophage; 1+ = minimal, 2+ = mild.

Table A.4. Bronchoalveolar Lavage Fluid Analyses for Rodents Exposed for 6 Hours to 18 ppm BDO_2

Days After Exposure	Animal Number	Treatment	White Blood Cells ($\times 10^5$)	Alveolar Macrophages ($\times 10^5$)	Polymorphonuclear Leukocytes ($\times 10^6$)	Protein (mg/mL)
Rats						
3	A-001	Control	12.08	11.64	0.00	0.071
	A-003	Control	15.92	14.88	0.00	0.061
	A-004	Control	6.44	6.13	0.00	0.112
0	A-005	Exposed	6.01	6.01	0.00	0.133
	A-006	Exposed	9.71	9.35	0.00	0.085
	A-007	Exposed	6.11	6.11	0.00	0.073
1	A-008	Exposed	32.69	31.43	0.31	0.190
	A-010	Exposed	12.93	12.80	0.00	0.112
	A-011	Exposed	12.48	12.38	0.00	0.063
3	A-012	Exposed	10.80	10.80	0.00	0.086
	A-013	Exposed	10.43	9.48	0.00	0.100
	A-014	Exposed	17.00	16.75	0.00	0.126
Mice						
3	B-003	Control	2.24	2.20	0.00	0.968
	B-004	Control	1.80	1.69	0.00	0.675
0	B-005	Exposed	2.40	2.34	0.00	0.457
	B-006	Exposed	3.00	2.89	0.03	0.386
	B-007	Exposed	1.08	1.05	0.00	0.205
	B-008	Exposed	4.60	4.46	0.00	0.354
1	B-009	Exposed	3.17	3.04	0.00	0.454
	B-010	Exposed	1.41	1.41	0.00	0.420
	B-011	Exposed	1.61	1.52	0.00	0.366
	B-021	Exposed	8.75	7.57	0.07	0.570
3	B-012	Exposed	1.50	1.50	0.00	0.252
	B-013	Exposed	1.49	1.39	0.01	0.194
	B-014	Exposed	2.01	2.01	0.00	0.538
	B-015	Exposed	0.56	0.50	0.00	0.217

20 ppm BDO_2. The lower level provided a backup in case the 20 ppm level proved to be too high and caused premature deaths. The lower level also provided mice with lung doses approximately equal to those of the rats exposed to higher levels, so that the responses of the two species to approximately the same dose could be compared.

Experimental Design

The design was similar to that in the original proposal except that the mice and rats were exposed by inhalation 6 hours/day, 5 days/week for 4 weeks. There were six exposure groups including rats or mice exposed to air, 10 ppm, or 20 ppm BDO_2 in whole-body chambers. The number of rodents in each exposure group was increased from 48 to 56 rats or mice. The additional eight rodents in each group were used for an end-of-exposure observation group, in which blood was taken during necropsy via cardiac puncture for analysis of the hematologic parameters, instead of using retro-orbital bleeding; this change was requested by our Animal Care Committee. We also performed a histopathological evaluation of these animals to document the immediate effect of the 4-week exposures to BDO_2.

The rest of the animals were held for later histopathologic examination, with most to be examined at 12 months after exposure. Animals were weighed monthly as an indicator of toxicity. Four sentinel animals were examined at 6 months; if the animals already had multiple tumors, the other animals were killed earlier than planned. If no tumors were observed

at 6 months, another set of four sentinel animals were scheduled for observation at 12 months after the exposure.

ABOUT THE AUTHORS

Rogene F. Henderson was a Fulbright Scholar in physical chemistry (Munich), earned a Ph.D. in chemistry at the University of Texas in 1960, and performed postdoctoral research at the University of Arkansas School of Medicine. She is a senior scientist at the Lovelace Respiratory Research Institute as well as deputy director of the National Environmental Respiratory Center. She has also served as a member of the National Institutes of Health Toxicology Study Section and the National Institute of Environmental Health Sciences Advisory Council and has been chairman of the National Academy of Sciences/National Research Council Committee on Toxicology. Dr. Henderson's research interests include the toxicokinetics of inhaled vapors and gases, the development of lavage techniques as probes to detect lung injury, and the use of chemical biomarkers.

Edward B. Barr, an aerosol scientist, received his M.S. in electrical engineering from the University of New Mexico in 1977. He has worked at the Lovelace Respiratory Research Institute since 1971. His research interests are in aerosol generation and characterization as applied to multichamber exposure systems, the development of powder generation techniques, and computer monitoring and control of exposure systems.

Steven A. Belinsky received his Ph.D. in toxicology from the University of North Carolina in 1984 and is currently head of the Lung Cancer Program at the Lovelace Respiratory Research Institute. His research focuses on identifying factors in cell transformation by investigating changes in gene expression, activation of protooncogenes, and inactivation of tumor suppressor genes as well as DNA methylation in the control of gene transcription and expression.

Janet M. Benson earned her Ph.D. in comparative pharmacology and toxicology from the University of California, Davis, in 1978 and has worked at the Lovelace Respiratory Research Institute since 1977. She is now a senior scientist and manager of the Applied Toxicology Program. Dr. Benson's research interests lie in evaluation of the inhalation toxicity and toxicokinetics of gases, vapors, and particles. Current research focuses on rates of uptake and transport of iron from the respiratory tract, effects of inhaled metals on cardiac function in dogs, and the mechanisms of beryllium-induced granulomatous lung disease.

Fletcher F. Hahn earned his D.V.M. from Washington State University in 1964 and his Ph.D. in comparative pathology from the University of California, Davis, in 1971. He has worked at the Lovelace Respiratory Research Institute since 1971 where he is a senior scientist and experimental pathologist. His research interests focus on biological effects of inhaled environmental contaminants. Dr. Hahn has studied the pathogenesis and morphologic changes in pulmonary diseases of laboratory animals as well as the pulmonary inflammation, fibrosis, and neoplasia resulting from inhaled fibers, metallic particles, and oxidant gases.

Margaret G. Ménache earned her Ph.D. from Duke University in 1997. She is a statistician at the Lovelace Respiratory Research Institute with research interests in a broad range of classical statistical techniques, including parametric and nonparametric methods. She works with data that consist of discrete and continuous dependent variables (single or multiple) and is familiar with methods to analyze continuous, discrete, and mixed independent variables. Dr. Ménache is currently working on an empirical model to predict effects of mixtures of dioxin-like compounds.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Henderson RF, Hahn FF, Barr EB, Belinsky SA, Ménache MG, Benson JM. 1999. Carcinogenicity of inhaled butadiene diepoxide in female B6C3F$_1$ mice and Sprague-Dawley rats. Toxicol Sci 52:33–44.

Henderson RF, Hahn FF, Benson JM, Barr EB, Bechtold WE, Burt DG, Dahl AR. 1999. Dosimetry and acute toxicity of inhaled butadiene diepoxide in rats and mice. Toxicol Sci 51:146–152.

ABBREVIATIONS AND OTHER TERMS

ANOVA	analysis of variance
BD	1,3-butadiene
BDO	1,2-epoxy-3-butene
BDO_2	1,2,3,4-diepoxybutane
GC	gas chromatography
GSH	reduced glutathione
GST	glutathione *S*–transferase
MANOVA	multivariate analysis of variance
PCR	polymerase chain reaction
SPR1	small proline-rich protein

COMMENTARY ON PART I
Health Review Committee

HEI

INTRODUCTION

Long-term inhalation of 1,3-butadiene (BD)* causes tumors at multiple sites in mice and rats, with mice being the more sensitive species (National Toxicology Program 1984, 1993; Owen et al. 1987; reviewed by Himmelstein et al. 1997). Interested readers can find summaries of these studies in the Preface to this Research Report and in the Introduction to the Investigators' Report. Butadiene is not a direct-acting rodent carcinogen. As shown in Figure 1 in the Preface, it is enzymatically oxidized to 1,2-epoxy-3-butene (BDO), which in turn can be oxidized to 1,2,3,4-diepoxybutane (BDO_2). BDO_2 can be converted enzymatically to 1,2-dihydroxy-3,4-epoxybutane (BDO-diol). Although Cochrane and Skopek (1994) demonstrated that BDO_2 was more mutagenic in vitro than BDO or BDO-diol, researchers have not yet determined which of the BD metabolites is the proximate in vivo carcinogen in mice and rats. Moreover, the reactive metabolites can be enzymatically detoxified to different degrees in mice and rats, leading some researchers to propose that the balance of metabolic activation and inactivation may be responsible for the lower level of BD-induced tumorigenicity in rats (reviewed by Himmelstein et al. 1997 and discussed in the Investigators' Report).

In response to RFA-93-1, *Novel Approaches to Extrapolation of Health Effects for Mobile Source Toxic Air Pollutants*, Dr. Rogene F. Henderson, of the Lovelace Respiratory Research Institute, submitted an application entitled "Study of the Potential Carcinogenicity of Butadiene Diepoxide [BDO_2] in Mice and Rats."† The following Commentary is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study, by pointing out alternative explanations for the results, and by placing the Investigators' Report into scientific and regulatory perspective.

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

† Dr. Henderson's 2.5-year study began in June 1995 and had total expenditures of $238,300. During her study, Dr. Henderson collaborated with other investigators in HEI's butadiene program by exposing additional rats and mice to BD, BDO, or BDO_2. (The results of these exposures are included in the reports by Drs. Recio and Walker.) The Investigators' Report from Dr. Henderson and colleagues was received for review in July 1998. A revised report, received in October 1998, was accepted for publication in December 1998. During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary.

OBJECTIVES AND STUDY DESIGN

The purpose of Dr. Henderson's study was to determine whether BDO_2 is equally tumorigenic in mice and rats when administered at levels that produce equivalent internal doses. If BDO_2 is the proximate carcinogen that causes a similar incidence of neoplasms in rats and mice when administered at similar doses, it would be reasonable to infer that the carcinogenicity of BD in humans is based upon the propensity for human cells to convert BD to BDO_2. The HEI Health Research Committee thought that Henderson's proposal addressed this important question in a straightforward manner and that the study's results would help in interpreting the results of other studies in HEI's research program on BD. The specific objectives of this project were twofold:

1. To determine the carcinogenic response of female $B6C3F_1$ mice and Sprague-Dawley rats to repeated inhalation exposures to BDO_2. Females were chosen because of their greater susceptibility to BD-induced carcinogenesis.
2. To assay for mutations to K-*ras* codon 13 as a marker of BD-specific mutations and determine if BDO_2 induces DNA adducts leading to these mutations. The rationale behind this aim was that BD-induced lung tumors in mice contain an activated K-*ras* gene (Goodrow et al. 1990).

The investigators exposed six-week-old mice and rats to a racemic mixture of BDO_2 [equal amounts of the (*R*,*R*) and (*S*,*S*) stereoisomers with no *meso* form]. In preliminary experiments, Henderson evaluated intraperitoneal administration, intratracheal instillation, and nose-only inhalation routes of exposure and established inhalation as the preferred route to achieve the desired blood and tissue concentrations of BDO_2.‡ Subsequent inhalation exposures of rats and mice to 10 or 20 parts per million (ppm) BDO_2 (6 hours/day, 5 days/week for 4 weeks) revealed a higher level of toxicity than predicted. As a result, Henderson and colleagues changed their study design and exposed groups of 56 mice and 56 rats to 0, 2.5, or 5.0 ppm BDO_2 for 6 hours/day, 5 days/week, for 6 weeks. They planned to kill several animals from each group at the end of the exposure period (to determine the acute toxicity of BDO_2) and at 6 and 12 months after exposure. The results of these latter analyses were intended to help estimate

‡ These studies were supported by the Chemical Manufacturers Association and are included as an Appendix to the Investigators' Report.

when tumor development would be optimal after cessation of exposure and therefore when animals should be killed to gather the most complete data. Animals were killed at the end of exposure as planned; however, because a large number of rats (including control rats exposed to 0 ppm BDO_2) died after exposure ceased, the investigators did not kill rats on the designed interim schedule. A group of mice were killed at 6 months and no animals were killed at 12 months. In addition to those killed on schedule, mice and rats died or became moribund after exposure ceased. The latter were euthanized and both groups were analyzed as "unscheduled deaths." The investigators decided to conduct their final analyses on the remaining animals 18 months after exposure. The large number of unscheduled rat deaths forced them to change their original aim of comparing BDO_2 carcinogenicity between species to within-species comparisons only. They evaluated hematologic parameters at the end of exposure and at 18 months, and nonneoplastic and neoplastic lesions by histopathology im- mediately after exposure, at the time of unscheduled deaths, and at the final 18-month time point.

TECHNICAL EVALUATION

METHODS

A strength of this study was the precisely controlled inhalation exposures performed by an experienced group of scientists. The methods for determining hematologic and histopathologic endpoints after BDO_2 exposure were adequate and appropriate to their aims. The statistical analyses, although appropriate, were different from those originally planned because of the unscheduled rat deaths.

RESULTS

At the end of the BDO_2 exposure period, rats had no biologically significant alterations in standard hematologic parameters. The number of red blood cells was elevated 18 months after exposure but this was not dose dependent. Mice had a dose-dependent increase in blood neutrophils and a decrease in lymphocytes immediately after exposure; both changes were less severe 18 months later.

The most significant lesions in both species were in the nose, concentrated around the main airflow pathway. At the end of exposure to 5.0 ppm BDO_2, both species showed necrosis, inflammation, squamous metaplasia of the nasal mucosa, and atrophy of the turbinates. Squamous metaplasia persisted in both species; in some rats it was severe enough to restrict or occlude the nasopharyngeal duct. These lesions were less severe and less frequent in rats and mice exposed to 2.5 ppm BDO_2. Keratinizing squamous cell carcinomas developed within the metaplastic foci in rats, but not in mice. At 18 months after exposure, a dose-dependent increase in neoplasms of the nasal mucosa was evident in rats but not in mice. (This latter finding was also characteristic of rats and mice analyzed as unscheduled deaths.) Also at 18 months, no significant increases in neoplasms were noted at other sites in rats; however, mice had neoplastic lesions in reproductive organs, lymph nodes, liver, Harderian gland, pancreas, and lungs. The total number of neoplastic lesions in all organs in mice showed a dose-dependent and statistically significant increase; however, the only individual organ that showed a significant increase was the Harderian gland.

The investigators were unable to analyze mutations of the K-*ras* gene in the tumors arising from the nasal mucosa as originally planned, presumably due to hydrolysis of DNA during the fixation of the tissue for histopathology. If the investigators had conducted mutational and adduct analyses in tissues and tumors from exposed animals, they may have obtained information as to whether adducts formed in an expected target tissue, such as the mouse lung. If this were the case, such information might indicate that the lack of significant tumor formation was more a result of the biology of carcinogenesis in the mouse than due to the toxicokinetic parameters of the agent.

INTERPRETATIONS AND IMPLICATIONS FOR FUTURE RESEARCH

Henderson and coworkers clearly established that the racemic mixture of BDO_2 is carcinogenic in rats and mice. Because BDO_2 was expected to be a direct-acting carcinogen, active at sites of administration, the high incidence of carcinomas of the nose (the site of administration) in rats is understandable. However, exposure to BDO_2 did not cause tumors at the same target sites as the parent molecule, BD, is known to do. In particular, lung tumors, which result from inhaling BD, were not found in mice exposed to BDO_2, even though Henderson's preliminary studies (see Appendix A in the Investigators' Report) indicated that mice had higher levels of BDO_2 after inhalation exposure than rats. Thus, inhalation of BDO_2 may not be equivalent to exposure to its parent compound.

The lack of nasal tumors in mice exposed to BDO_2 is perplexing in view of tumor formation at internal sites. Some researchers suggest that the striking difference between rats and mice in their carcinogenic response to BD exposure is related to differences in BD metabolism (reviewed by Himmelstein et al. 1997). Because the mice

were not intrinsically resistant to BDO_2, as evidenced by the appearance of tumors at sites other than the nose, differences in the metabolism of BD to BDO_2 do not satisfactorily explain the marked species differences in carcinogenic susceptibility to the parent compound. It is plausible that systemic administration of BDO_2 did not replicate the concentrations of BDO_2 generated at target tissues by in situ BD metabolism in other studies. For example, inhaled BDO_2 must traverse the cell cytoplasm to reach target sites in cell nuclei, and cytoplasm contains enzymes that detoxify BDO_2. In addition, other factors, such as the differential toxicity of the stereochemical forms produced by BD metabolism, may contribute to species differences in BDO_2 carcinogenicity. (Such factors could also be relevant to human risk.) The stereochemical form of BDO_2 influences its metabolism and toxicity (Nieusma et al. 1997); therefore, it is reasonable to speculate that the stereochemical form also will influence its carcinogenicity. The stereochemical form of other epoxide carcinogens (e.g., polynuclear aromatic hydrocarbons) impacts their activities in cancer bioassays (Butch et al. 1992; Reddy et al. 1992; Lau et al.1995; Ralston et al.1995; Melendez-Colon et al. 1997).

The investigators recognized limitations in using a racemic mixture of BDO_2 stereoisomers because it is not known how closely this corresponds to the mixture of stereoisomers produced in target tissues by in situ metabolism of BD. Thus, the results must be interpreted cautiously as representing the carcinogenicity of the racemic mixture, but not necessarily of the metabolic products of BD. Although the results do not firmly establish the precise role of BDO_2 in BD-induced carcinogenicity, neither do they provide evidence to suggest that BDO_2 is not involved in this process. They also complement the findings of Recio and Walker (also published in this Research Report) on the mutagenicity of the same racemic mixture.

Future research to determine which stereochemical form or forms predominate in laboratory animals exposed to BD might include analyses of BD-DNA adducts after exposure to individual stereoisomers of BDO_2, and analysis of mutational spectra (specific changes in the sequence of DNA bases). The latter could be examined in reporter genes (genes that are not affected by carcinogenesis but are believed to mirror changes in affected genes), or in oncogenes (genes capable of causing the initial and continuing conversion of normal cells to cancer cells) in target tissues. (The Recio and Walker studies employed reporter genes.) Comparing this information with the adduct and mutation patterns found in DNA of humans exposed in the workplace should increase our understanding of the risk of exposure to BD by giving us insight into species differences.

SUMMARY

Henderson and colleagues studied the potential carcinogenicity of the BD metabolite, BDO_2. Rats and mice exposed by inhalation to 5.0 ppm of a racemic mixture of BDO_2 stereoisomers developed significant lesions that were concentrated around the main airflow pathway of their nasal passages. Rats, but not mice, showed a dose-dependent increase in nasal tumors 18 months after exposure ceased. No significant increases in tumors were found at other sites in rats. A dose-dependent and statistically significant increase was noted in the total number of tumors in mouse tissues (reproductive organs, lymph nodes, liver, Harderian gland, pancreas, and lung), but the Harderian gland was the only individual tissue to show a significant increase in tumors.

The results of this study by Henderson and colleagues must be interpreted cautiously as representing the carcinogenicity of the racemic mixture of BDO_2. Because it is not known how closely this corresponds to the mixture of stereoisomers produced in target tissues by metabolism of BD in rats and mice, the results do not firmly establish the precise role of BDO_2 in BD-induced carcinogenicity. However, the results also do not rule out the possibility that BDO_2 is somehow involved in this process.

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REFERENCES

Butch ER, Lau HH, Shaw KL, Smolarek TA, Schmerold TA, Anderson JN, Baird WM, Yagi H, Jerina DM. 1992. High selectivity of polyclonal antibodies against DNA modified by diastereomeric benzo[*c*]phenanthrene-3,4-diol-1,2-epoxides. Carcinogenesis 13:895-899.

Cochrane JE, Skopek TR. 1994. Mutagenicity of butadiene and its epoxide metabolites: I. Mutagenic potential of 1,2-epoxybutene, 1,2,3,4-diepoxybutane and 3,4-epoxy-1,2-butanediol in cultured human lymphoblasts. Carcinogenesis 15:713-717.

Goodrow T, Reynolds S, Maronpot R, Anderson M. 1990. Activation of K-*ras* by codon 13 mutations in C57BL/6 C3H F_1 mouse tumors induced by exposure to 1,3-butadiene. Cancer Res 50:4818-4823.

Himmelstein MW, Acquavella JF, Recio L, Medinsky MA, Bond JA. 1997. Toxicology and epidemiology of 1,3-butadiene. Crit Rev Toxicol 27:1-108.

Lau HH, Coffing SL, Lee H, Harvey RG, Baird WM. 1995. Stereoselectivity of activation of 7,12-dimethylbenz[*a*]anthracene-3,4-dihydrodiol to the anti-diol epoxide metabolite in a human mammary carcinoma MCF-7 cell-mediated V79 mutation assay. Chem Res Toxicol 8:970-978.

Melendez-Colon VJ, Smith CA, Seidel A, Luch A, Platt KL, Baird WM. 1997. Formation of stable adducts and absence of depurinating DNA adducts in cells and DNA treated with the potent carcinogen dibenzo[*a,l*]pyrene or its diol epoxides. Proc Natl Acad Sci USA 94:13542-13547.

National Toxicology Program. 1984. Toxicology and Carcinogenesis Studies of 1,3-Butadiene (CAS No. 106-99-0) in B6C3F_1 Mice (Inhalation Studies) pp. 1-111. NTP TR No. 288. National Toxicology Program, Research Triangle Park, NC.

National Toxicology Program. 1993. NTP Technical Report on the Toxicology and Carcinogenesis Studies of 1,3-Butadiene, pp. 1-391. NTP TR 434, No. 93-3165. National Toxicology Program, Research Triangle Park, NC.

Nieusma JL, Claffey DJ, Maniglier-Poulet C, Imiolczyk T, Ross D, Ruth JA. 1997. Stereochemical aspects of 1,3-butadiene metabolism and toxicity in rat and mouse liver microsomes and freshly isolated rat hepatocytes. Chem Res Toxicol 10:450-456.

Owen PE, Glaister JR, Gaunt IF, Pullinger DH. 1987. Inhalation toxicology studies with 1,3-butadiene: 3. Two year toxicity/carcinogenicity study in rats. Am Ind Hyg Assoc J 48:407-413.

Ralston SL, Seidel A, Luch A, Platt KL, Baird WM. 1995. Stereoselective activation of dibenzo[*a,l*]pyrene to (-)-*anti*-(11*R*,12*S*,13*S*,14*R*)- and (+)-*syn*-(11*S*,12*R*,13*S*,14*R*)-11,12-diol-13,14-epoxides which bind extensively to deoxyadenosine residues of DNA in the human mammary carcinoma cell line MCF-7. Carcinogenesis 16:2899-2907.

Reddy AP, Preuss-Schwartz D, Ji C, Gorycki P, Marnett LJ. 1992. ^{32}P-Postlabeling of DNA adduction in mouse skin following topical administration of (+)-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene. Chem Res Toxicol 5:26-33.

1,3-Butadiene: Cancer, Mutations, and Adducts

Part II: Roles of Two Metabolites of 1,3-Butadiene in Mediating Its in Vivo Genotoxicity

Leslie Recio, Christopher J. Saranko, and Ann-Marie Steen

ABSTRACT

1,3-Butadiene (BD)* is carcinogenic in mice and rats, with mice being more susceptible than rats to its carcinogenic effects. 1,3-Butadiene is mutagenic in the bone marrow and spleen cells of B6C3F$_1$ *lacI* transgenic mice. The goal of this research was to assess the roles of two BD metabolites, 1,2-epoxy-3-butene (BDO) and 1,2,3,4-diepoxybutane (BDO_2), in the mutagenicity and mutational spectrum of the parent compound BD by determining the mutagenicity and mutational spectra of BDO and BDO_2 in human and rodent cells in vitro and in vivo. In human TK6 lymphoblastoid cells (TK6 cells), BDO exposure increased the frequency of G•C→A•T transitions and A•T→T•A transversions (Fisher exact test; $p < 0.05$). The most striking difference in the type of base-substitution mutations between BDO-exposed and BDO-unexposed TK6 cells was the 19-fold increase in A•T→T•A transversions. 1,2,3,4-Diepoxybutane increased the frequency of A•T→T•A transversions (Fisher exact test; $p < 0.05$) and the frequency of deletions in exposed TK6 cells compared with unexposed controls. Exposure of Rat2 *lacI* transgenic fibroblasts (Rat2 cells) to BDO increased the frequency of three types of base-substitution mutations: G•C→A•T transitions, G•C→T•A transversions, and A•T→T•A transversions. Exposure of Rat2 cells to BDO_2-induced dose-dependent increases in micronuclei at exposure levels that apparently did not induce mutagenicity at the *lacI* transgene. The lack of detectable mutagenicity at the *lacI* transgene in Rat2 cells exposed to BDO_2 probably reflects the poor recovery of large deletions by this lambda phage–based mutagenicity assay. Inhalation exposure of B6C3F$_1$ *lacI* transgenic mice (*lacI* mice) and F344 *lacI* transgenic rats (*lacI* rats) to BDO (29.9 parts per million [ppm]; 6 hours/day; 5 days/week for 2 weeks) did not increase the *lacI* mutant frequency (MF) in bone marrow or spleen cells of mice and rats, but in the cells of mouse lung (a tumor target organ for BD), significant mutagenicity was observed. An increased *lacI* MF was also observed in the bone marrow cells of rats exposed to BDO. Inhalation exposure of *lacI* mice and *lacI* rats to BDO_2 (3.8 ppm; 6 hours/day; 5 days/week for 2 weeks) did not increase the *lacI* MF in bone marrow or spleen cells of mice or in the spleen cells of rats. An increased *lacI* MF was observed in the bone marrow cells of rats exposed to BDO_2.

In the present study, BDO specifically induced G•C→A•T and A•T→T•A transversions in vitro at both the endogenous hypoxanthine phosphoribosyltransferase (*hprt*) gene and the *lacI* transgene in Rat2 cells. It also induced an increased frequency of G•C→T•A transversions in Rat2 cells. These types of mutations also occur at an increased frequency in mice exposed to the parent compound, BD. This finding demonstrates the induction of consistent mutational types across biological systems by BDO and indicates that BDO, but not BDO_2, probably has a role in mediating the mutations recovered at the *lacI* transgene in animals exposed to the parent compound, BD. Therefore, it is apparent that in mice exposed to BD at carcinogenic levels, BDO and BDO_2 act in concert to mediate the range of genotoxic responses. These data demonstrate that certain DNA adducts (guanine or adenine) may be useful biomarkers for BD genetic effects. However, other DNA lesions that can account for BDO_2-induced deletions and chromosomal alterations also need to be considered as biomarkers for BD-induced genotoxicity.

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

This Investigators' Report is Part II of Health Effects Institute Research Report Number 92, which also includes four additional Investigators' Reports (Parts I and III through V) by different research groups, a Commentary by the Health Review Committee on Parts II and III, a Commentary on Part I, a Commentary on Parts IV and V, and an HEI Statement about the five butadiene research projects reported here. Correspondence concerning the Part II Investigators' Report may be addressed to Dr. Leslie Recio, Chemical Industry Institute of Toxicology, 6 Davis Drive, P.O. Box 12137, Research Triangle Park, NC 27709-2137.

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INTRODUCTION

1,3-Butadiene is used in the production of synthetic rubber and has a worldwide annual consumption of 6.1 million metric tons (DeWitt & Co. 1996). It is among the top 50 chemicals produced in the U.S. (Chemical and Engineering News 1994) and is regulated as a hazardous air pollutant under the 1990 Clean Air Act Amendments (U.S. Environmental Protection Agency 1991). 1,3-Butadiene is carcinogenic in rats and mice. However, there are striking species differences in cancer susceptibility and the spectrum of tumors between mice and rats, with mice being more susceptible to tumor induction than rats (National Toxicology Program 1984; Huff et al. 1985; Melnick et al. 1990a,b; Owen and Glaister 1990). According to Melnick and colleagues (1990a,b), $B6C3F_1$ mice (females) develop significant increases in lung tumors with BD exposure at 6.25 ppm, whereas rats develop tumors at concentrations of 1,000 or 8,000 ppm, which are as much as three orders of magnitude higher than those that cause cancer in mice (6.25 to 1,250 ppm). In mice, BD induces primarily lymphomas, lung tumors, and liver tumors (Melnick et al. 1990a,b). In rats, BD induces primarily mammary-gland tumors and pancreatic tumors (Owen and Glaister 1990).

The toxicology and epidemiology of BD have been the subject of a number of reviews and discussions (Adler et al. 1995a; Jacobson-Kram and Rosenthal 1995; Melnick and Kohn 1995; Bond et al. 1995; Sorsa et al. 1996). A detailed review of BD toxicology, including epidemiology, metabolism, toxicokinetics, and genotoxicity, was recently published (Himmelstein et al. 1997). The International Agency for Research on Cancer (IARC) has classified BD as a Group 2A carcinogen (i.e., probable human carcinogen) (1992) on the basis of sufficient evidence of carcinogenicity in animals and limited evidence of carcinogenicity in humans. After extensive review and public comment, the Occupational Safety and Health Administration (OSHA) reduced the 8-hour time-weighted average permissible BD exposure level from 1,000 ppm to 1 ppm (1996). Epidemiologic studies show that, compared with the general population, workers in North American styrene-butadiene rubber plants have an increased risk of leukemia that appears to be associated with BD exposure (Delzell et al. 1996; Macaluso et al. 1996).

1,3-Butadiene is biotransformed in vitro and in vivo to a number of metabolites (reviewed in Himmelstein et al. 1997), three or more of which are directly mutagenic. Of these, the present studies focus on BDO and BDO_2 but do not examine 1,2-dihydroxy-3,4-epoxybutane (BDO-diol). This BD metabolite is also directly mutagenic in human cells (Cochrane and Skopek 1994a) but at concentrations that are 3.5- and 350-fold greater than the mutagenic concentrations of BDO and BDO_2, respectively. Blood levels of BDO-diol have not been quantified in animals following exposure to BD, and only two experiments have been performed in rats. In one of these, a single intraperitoneal dose of BDO-diol slightly increased the micronucleus frequency. In vivo pharmacokinetic data are lacking on BDO-diol levels in mice or rats following inhalation exposure. Genotoxicity data demonstrate that BDO-diol is a weak inducer of micronuclei in vivo (Lähdetie and Grawé 1997). For these reasons, we chose not to focus on BDO-diol in the present study.

Other potentially DNA-reactive metabolites may arise through the oxidation of BD to aldehydes such as 3-butenal, crotonaldehyde, and acrolein (Nauhaus et al. 1996). Although some of these aldehydes have been quantified in the urine of mice and rats, they have not been quantified by pharmacokinetic studies in mice and rats. Consistent mutagenicity data showing that 3-butenal, crotonaldehyde, and acrolein are mammalian-cell mutagens are not available (IARC 1995). In general, this class of carcinogenic DNA-reactive aldehydes is cytotoxic and weakly mutagenic in mammalian cells (Feron et al. 1991). For example, acrolein is cytotoxic but not mutagenic at *hprt* in Chinese hamster V79 cells (Parent et al. 1991). The IARC has reviewed the available data and concluded that there is inadequate evidence in laboratory animals or in humans to demonstrate the carcinogenicity of crotonaldehyde or acrolein. Due to the relatively weak genotoxicity exhibited by DNA-reactive aldehydes, the lack of convincing evidence for carcinogenicity in either animals or in humans, and the lack of pharmacokinetic data on BD-derived aldehydes, we did not examine the mutagenicity of these metabolites. The genotoxicity of DNA-reactive aldehydes is less than the genotoxic properties exhibited by the epoxide metabolites of BD. This suggests that BD-derived DNA-reactive aldehydes do not have a significant role in the induction of in vivo genotoxicity and perhaps carcinogenicity in mice from exposure to BD.

The production of the DNA-reactive metabolites BDO and BDO_2 from BD is hypothesized to have a causal role in tumor development from BD exposure by inducing genetic alterations (mutations) in genes believed to be involved in the development of cancer (Figure 1). These epoxide metabolites show species differences in blood levels following inhalation exposure to BD that are consistent with cancer susceptibility; they are direct mutagens at micromolar concentrations, as shown by a wide variety of test systems, and can directly induce tumors at the site of injection in mice (Himmelstein et al. 1997). The overall objective of the present studies was to examine the mutational spectra of BDO and BDO_2 in vitro and in vivo to assess mechanisms of mutation and the roles of BDO and BDO_2 in mediating the in vivo mutagenicity of the parent

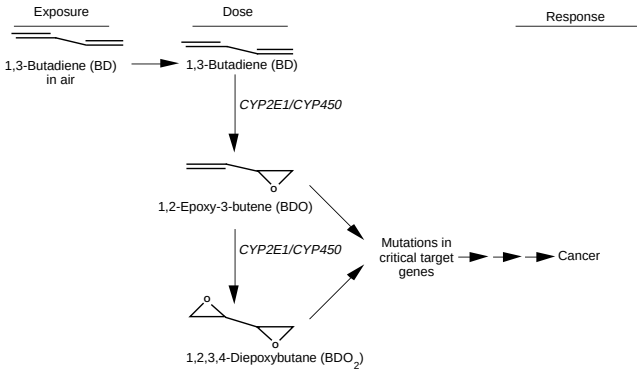

Figure 1. Results of exposure to BD in air. BD is absorbed into the body and metabolized to at least two DNA-reactive metabolites, BDO and BDO_2. This bioactivation is catalyzed by the P450 isozyme *CYP2E1*. These metabolites can induce a wide variety of genetic damage, resulting in mutations that can ultimately lead to the induction of cancer in rodents. The levels of BDO and BDO_2 are greater in mice than in rats and are consistent with differences in cancer potency between these two species. (Adapted from Recio et al. 1997 with permission from the Chemical Industry Institute of Toxicology.)

compound BD. The mutagenicity and mutational spectra of BDO and BDO_2 were examined at the endogenous *hprt* gene in cultured human TK6 cells, at the *lacI* transgene incorporated into the genome of cultured rodent cells (Rat2 fibroblasts), and at the *lacI* transgene incorporated into the genome of mice and rats.

BIOACTIVATION OF BD TO BDO AND BDO_2

The bioactivation of BD to BDO and BDO_2 can be catalyzed by P450 isozymes present in mouse, rat, and human liver microsomes (Csanády et al. 1992). Overall, mouse liver microsomes have faster rates of BD oxidation than rat or human liver microsomes. Among purified P450 isozymes derived from cDNA-expressed cytochrome P450s, *CYP2A6*, *CYP2E1*, and *CYP3A4* have been identified as P450 isozymes with activity for the oxidation of BDO to BDO_2 (Duescher and Elfarra 1994; Seaton et al. 1995). Detailed kinetic studies suggested that *CYP2E1* was responsible for both the oxidation of BD to BDO at low BD concentrations and for the conversion of BDO to BDO_2 at low BDO concentrations. Therefore, *CYP2E1* appears to be the principal enzyme responsible for the metabolism of BD to BDO and BDO to BDO_2 at the low exposure concentrations of BD expected to occur in an occupational or environmental setting (Figure 1).

In vivo data on BD metabolism in mice and rats indicate that the blood levels of BDO and BDO_2 are greater in mice than in rats (Himmelstein et al. 1997). Studies in male $B6C3F_1$ mice and male Sprague-Dawley rats exposed to BD (Himmelstein et al. 1994, 1995; Thornton-Manning et al. 1996) were conducted at inhalation concentrations corresponding to those used in the chronic bioassays for mice and rats (Melnick et al. 1990; Owen and Glaister 1990) to measure the in vivo concentrations of BD, BDO, and BDO_2 in blood, lung, and liver. At BD exposures of 62.5 ppm to 1,250 ppm, the blood levels of BDO in mice ranged from 0.56 µM to 8.6 µM, whereas the blood levels of BDO in rats ranged from 0.07 µM to 1.3 µM (Himmelstein et al. 1994). After BD exposure to 62.5 ppm, the blood level of BDO_2 in mice was 0.2 µM, whereas in rats it was 0.005 µM (Thornton-Manning et al. 1995). During BD exposure, blood concentrations of BDO were 4 to 8 times higher in mice than in rats; the blood concentrations of BDO_2 were 40 times higher in mice than in rats. These data demonstrate that mice experience higher levels of the DNA-reactive metabolites BDO and BDO_2 than do rats.

In vitro studies of BD metabolism indicate that mice form the DNA-reactive metabolites BDO and BDO_2 at a faster rate than do rats or humans (Csanády et al. 1992). In vivo studies of the metabolism and tissue concentrations of BDO and BDO_2 in mice and rats following inhalation exposures to BD are consistent with in vitro studies on the metabolism of BD (Himmelstein et al. 1994, 1995; Thornton-Manning et al. 1995). The faster rate of BD metabolism to BDO and BDO_2 in mice compared to rats results in higher blood and tissue concentrations of these DNA-reactive metabolites, which probably contribute to the increased sensitivity of mice to the carcinogenic effects of BD inhalation. Because *CYP2E1* mediates the bioactivation of BD to BDO and BDO_2, this isozyme must also have a key role in mediating species differences in response to BD. The 60-fold variation among humans in its activity for converting BDO to BDO_2 (Seaton et al. 1995) suggests the potential for large interindividual variation in susceptibility to the potential genotoxic effects of BD.

GENOTOXIC AND MUTATIONAL EFFECTS OF BD, BDO, AND BDO_2

The genotoxic effects of BD, BDO, and BDO_2 have been reviewed by de Meester (1988), as part of the IARC evaluation of BD (IARC 1992), by Jacobson-Kram and Rosenthal (1995), and by Himmelstein and colleagues (1997). 1,3-Butadiene is genotoxic in a number of in vitro and in vivo experimental systems including bacterial cells, yeast, *Drosophila*, mammalian cells in culture (including human cells), and animals (mice) (IARC 1992; Adler et al. 1995a,b,c; Jacobson-Kram and Rosenthal 1995; Himmelstein et al. 1997). BD is also a germ-cell mutagen in mice, inducing dominant lethal and heritable translocations following inhalation exposure (Adler and Anderson 1994; Adler et al. 1994, 1995b,c).

The BD metabolites BDO and BDO_2 have different mechanisms of DNA interaction, which probably mediate their differences in genotoxicity. BDO is a monofunctional alkylating agent, whereas BDO_2 is a bifunctional alkylating

agent that can induce DNA interstrand cross links between adjacent guanine bases (Lawley and Brooks 1963, 1967). A variety of DNA adducts have occurred following in vitro or in vivo exposure to BD, BDO, or BDO_2. Adenine and guanine adducts have been observed after BD exposures in both in vitro and in vivo experimental systems (Citti et al. 1993; Koivisto et al. 1995, 1997; Leuratti et al. 1994; Neagu et al. 1995; Selzer and Elfarra 1996; Tretyakova et al. 1996, 1997). Following inhalation exposure of rats to BD, N6-adenine and N7-guanine adducts have been detected in liver cells (Sorsa et al. 1996; Koivisto et al. 1997). In one study, six adducts (four at adenine and two at guanine) were detected following reaction of BDO with DNA isolated from calf thymus cells (Tretyakova et al. 1996, 1997). Reaction of BDO with guanosine resulted in eight guanosine adducts that consisted of four diastereomeric pairs (Selzer and Elfarra 1996). Reaction of BDO with thymidine produced four N3-thymidine adducts consisting of two diastereomeric pairs (Selzer and Elfarra 1997). Specific adenine and guanine adducts have also been described following in vitro reactions of BDO_2 with adenosine and guanosine (Leuratti et al. 1994; Tretyakova et al. 1996). Due to the numerous DNA adducts described from exposure to BD, BDO, or BDO_2, it is premature to invoke one or more specific adducts that could give rise to the range of genotoxic responses resulting from exposure to BD and its genotoxic metabolites.

Following inhalation, BD can induce genotoxicity in $B6C3F_1$ mice through multiple mechanisms, including chromosomal alterations and single base-pair (bp) substitutions. It induces micronuclei in $B6C3F_1$ mice but not in rats following inhalation exposures of concentrations up to 500 ppm BD (Cunningham et al. 1986; Autio et al. 1994). Inhalation exposure of mice to BD at concentrations of 6.25 ppm, 62.5 ppm, or 625 ppm (6 hours/day; 5 days/week for 2 weeks) results in significant increases in a battery of genotoxic endpoints (Tice et al. 1987). For example, exposure to 6.25 ppm BD for 13 weeks increased the frequency of micronuclei in peripheral blood (Jauhar et al. 1988), as did a single 8-hour exposure to 62.5 ppm BD (Leavens et al. 1997). The increased susceptibility to the genotoxic effects (i.e., micronuclei) of BD in mice compared to rats is probably due to the increased blood levels of BDO and BDO_2 in mice compared to rats. These data indicate that an in vivo mechanism of BD-induced genotoxicity in exposed mice occurs through chromosomal alterations. The species differences in BD induction of tumors, in mice compared to rats, is paralleled by species differences in the induction of chromosomal alterations. There are no reports in the literature demonstrating an increased frequency of chromosomal alterations in rats exposed to BD.

Following inhalation exposure, BD induces in vivo gene mutation at marker genes in the tissues of transgenic mice and at the endogenous *hprt* gene in T lymphocytes of mice. Exposure of $B6C3F_1$ *lacI* transgenic mice to BD at 62.5 ppm, 625 ppm, or 1,250 ppm for 4 weeks (6 hours per day, 5 days per week) resulted in a two- to fourfold increase in the *lacI* MF above the background in the bone marrow and spleen cells (Sisk et al. 1994; Recio et al. 1996, 1997). Significant BD mutagenicity was observed in bone marrow and spleen cells at 62.5 ppm, the lowest exposure level tested (Sisk et al. 1994; Recio et al. 1997). Exposure of $B6C3F_1$ mice to 625 ppm BD (6 hours per day, 5 days per week, for 2 weeks) resulted in an increased frequency of *hprt*-mutant T lymphocytes, as compared with controls exposed only to air (Cochrane and Skopek 1994b). This finding in mice ($[102/E1 \times C3H/E1]F_1$) was also observed following a 5-day exposure to 1,300 ppm BD (6 hours per day) (Tates et al. 1994). These data indicate that exposure of mice to BD by inhalation results in an increased frequency of gene mutations.

To develop an understanding of BD-induced mutational mechanisms, studies of mutational spectra in target genes have begun to determine the types of mutational events that occur following BD inhalation exposures. DNA sequence analysis of the *lacI* transgene recovered from the bone marrow and spleen cells has demonstrated differences in the mutational spectrum between BD-exposed mice and air-exposed controls. An increased frequency of point mutations is observed at A•T base pairs (primarily A•T→T•A transversions) in the *lacI* transgene recovered from the bone marrow cells of transgenic mice exposed to BD at either 625 ppm or 1,250 ppm (Sisk et al. 1994; Recio and Meyer 1995) and spleen cells of transgenic mice exposed to 1,250 ppm BD (Recio et al. 1997). In spleen cells, an increased frequency of specific point mutations at G•C base pairs (G•C→A•T transitions at non-CpG sites and G•C→T•A transversions) is also observed (Recio et al. 1998). These studies demonstrate that BD can induce specific in vivo point mutations at A•T and G•C base pairs.

Molecular analysis of genetic alterations in oncogenes and tumor suppressor genes in the tumor cells of BD-exposed mice can also provide insights into mechanisms of BD-induced mutagenesis in vivo. Point mutations in the K-*ras* oncogene have been observed in cells from lung and liver tumors of BD-exposed mice (Goodrow et al. 1990). Loss of heterozygosity on mouse chromosomes 4 and 11 was observed at a high frequency in cells from lung tumors and mammary tumors of BD-exposed mice (Wiseman et al. 1994; Zhuang et al. 1997). These data demonstrate at least two mechanisms of genotoxicity: (1) point mutation, and (2) loss of heterozygosity resulting from deletions, rearrangements, or chromosome loss. Both mechanisms are

consistent with the induction of micronuclei, chromosome aberrations, and specific-locus gene mutations of the type reported in $B6C3F_1$ mice exposed to BD (Tice et al. 1987; Cunningham et al. 1986; Adler et al. 1994; Autio et al. 1994; Cochrane and Skopek 1994a,b; Sisk et al. 1994; Recio and Meyer 1995; Recio et al. 1997).

Exposure of mammalian cells or rodents to either BDO or BDO_2 results in several types of chromosomal alterations. Following in vitro exposure of developing rat spermatids to BDO or BDO_2, only BDO_2 increased the frequency of micronuclei (Sjöblom and Lähdetie 1996). The intraperitoneal injection of BDO or BDO_2 induced micronuclei in splenocytes of mice and rats (Xiao and Tates 1995). Intraperitoneal injection of BDO in mice and rats induced approximately a sevenfold increase in micronuclei in mouse spleen cells and a two- to threefold increase in rat spleen cells. The increased induction of micronuclei in BDO-exposed mice, compared with rats, suggests that the conversion of BDO to BDO_2 is a factor. Xiao and Tates (1995) found that BDO_2 induced micronuclei in spleen cells in both mice and rats at lower doses than were used for BDO. Confirmed in a separate study (Anderson et al. 1997), this finding indicates that BDO_2 is more effective than BDO at inducing in vivo chromosomal alterations (micronuclei) in mice and in rats.

Comparative studies of the in vitro genotoxicity of BDO and BDO_2 have shown that BDO_2 is genotoxic at lower concentrations than those used to induce BDO genotoxicity. In Chinese hamster ovary cells, BDO_2 induced a significant increase in sister chromatid exchange at 0.1 µM, whereas BDO induced a similar increase at 1.0 µM (Sasiadek et al. 1991a). In studies of the in vitro induction of sister chromatid exchange in human lymphocytes by BDO and BDO_2, the lowest effective concentration was 25 µM for BDO and 0.5 µM for BDO_2 (Sasiadek et al. 1991b). The induction of micronuclei and specific chromosomal gains has been observed in human lymphocytes exposed to BDO or BDO_2 in culture (Xi et al. 1997). In human TK6 cells, BDO_2 was mutagenic at the *hprt* and thymidine kinase (*tk*) loci at concentrations that were 100- and 350-fold lower than similarly mutagenic concentrations of BDO and BDO-diol, respectively (Cochrane and Skopek 1994b). These data indicate that in vitro BDO_2 can induce a number of genetic alterations at concentrations that are 10- to 100-fold less than those required for BDO to induce a similar response. They also indicate that BDO_2 is genotoxic in vitro at concentration levels that occur in the blood of mice exposed to BD. Therefore mice exposed to BD by inhalation experience circulating levels of BDO_2 that clearly can induce gene mutation and chromosomal alterations in mammalian cells.

Before the present studies, only limited studies on the mutational spectra of BDO and BDO_2 have been published. According to Cochrane and Skopek (1994a), BDO_2 is mutagenic at the *hprt* and *tk* loci of human TK6 cells at concentrations about 100-fold lower than BDO. Southern blot analysis of *hprt* mutants isolated from BDO_2-exposed TK6 cells indicated that a substantial fraction of these mutants had a total loss of *hprt* hybridization (21%), whereas mutants isolated from BDO-exposed TK6 cells had no change in the *hprt* hybridization pattern compared with the wild type. However, the frequency of alterations detectable by Southern blot in spontaneous *hprt* mutants was not reported. These studies in TK6 cells (Cochrane and Skopek 1994a) also demonstrated that BDO_2 induced an increase in *tk* mutants with a phenotype (slow growth) that is associated with loss of heterozygosity at *tk* (Li et al. 1992). In studies of *Drosophila*, BDO_2 induced deletion events (50 bp to 8,000 bp) at the *rosy* locus (Reardon et al. 1987; Gay and Contamine 1993). Taken together, these data indicate that (1) a primary mode of BDO_2 genotoxicity is the induction of large deletions and rearrangements and (2) a primary mode of BDO genotoxicity is the induction of small deletions or point mutations.

The present studies were proposed in response to the Health Effects Institute's request for research on BD that would permit the development of biomarkers useful for molecular epidemiologic studies in human populations exposed to BD. Investigation of the mutagenicity and mutational spectra of BDO and BDO_2 can identify the types of mutation that can be induced by each metabolite. These data can then be used to direct studies to identify DNA adducts that are biologically relevant to the induction of mutation. Previously unreported, the spectra of base-substitution mutations induced in human cells by BDO and BDO_2 represented a data gap in developing an understanding of how such alterations could lead to tumors. The determination of the mutational spectra of BDO and BDO_2 at the endogenous *hprt* gene in cultured human cells was part of the present studies.

Although specific mutations have been observed in vivo at the *lacI* transgene in $B6C3F_1$ *lacI* transgenic mice exposed to BD by inhalation, their origin is uncertain and is one of the issues addressed in this work. The mutagenicity and mutational spectra of BDO and BDO_2 were assessed using the same target gene (*lacI*) used to assess the in vivo mutagenicity and mutational spectrum of the parent compound BD. The mutagenic activity and mutational spectra of BDO and BDO_2 at the *lacI* transgene were compared with that observed for BD to investigate their roles in the mutagenicity of BD. By integrating in vitro studies using human and rodent cells and in vivo studies in rodents, insights into BD mutagenicity can be assessed, and biomarkers of BD-induced mutation can be developed.

SPECIFIC AIMS

To determine the mutagenicity and the mutational spectra of BDO and BDO_2 in human and rodent cells and to assess the specific contribution of each to the in vivo mutagenicity and mutational spectrum of the parent compound, BD, the three specific aims of this project were to study:

1. in vitro mutagenicity and mutational spectra of BDO and BDO_2 at *hprt* in human TK6 cells;
2. in vitro mutagenicity and mutational spectra of BDO and BDO_2 at the *lacI* transgene in a rodent transgenic cell line (Rat2 *lacI* transgenic fibroblasts); and
3. in vivo mutagenicity of BDO and BDO_2 in $B6C3F_1$ *lacI* transgenic mice and F344 *lacI* transgenic rats.

METHODS AND STUDY DESIGN

SPECIFIC AIM 1. THE IN VITRO MUTAGENICITY AND MUTATIONAL SPECTRA OF BDO AND BDO_2 AT *hprt* IN HUMAN TK6 CELLS

Chemicals

A racemic mixture of BDO (98%) [CAS 930-22-3] was purchased from Aldrich (Milwaukee, WI), and a racemic mixture of BDO_2 (97%) [CAS 1464-53-5] from Sigma (St. Louis, MO). Ethyl methane sulfonate (EMS) (99%) [CAS 62-50-0] and dimethylsulfoxide (DMSO) were purchased from Sigma. All other chemicals and reagents were of the highest purity available and were purchased from appropriate vendors.

Cell Culture and Exposures

Human TK6 cells are a B-lymphoblastoid cell line described by Skopek and colleagues (1978) that do not contain active *CYP2E1* (Cochrane and Skopek 1994a). For the present study, TK6 cells were grown in RPMI 1640 (Gibco BRL, Gaithersburg, MD) supplemented with 2 mM L-glutamine, 60 IU/mL penicillin, 60 mg/mL streptomycin, and 10% heat-inactivated equine serum at 37°C and 5% CO_2. The TK6 cells were maintained by daily subculture at 4×10^5 cells/mL.

Before exposure to BDO or BDO_2, TK6 cells were treated for 2 days with CHAT-supplemented media (CHAT: cytidine [10^{-5} M], hypoxanthine [2×10^{-4} M], aminopterin [2×10^{-7} M], thymidine [1.75×10^{-5} M]) to reduce the background *hprt* MF. They were subsequently grown in THC-supplemented media (THC: thymidine [10^{-5} M], hypoxanthine [2×10^{-4} M], cytidine [10^{-5} M]) and allowed to expand for 2 days.

TK6 cells were exposed to a racemic mixture of BDO (CAS 930-22-3; Sigma Chemical Co., St. Louis, MO) or BDO_2 (CAS 298-18-0; Sigma) stereoisomers in capped flasks containing complete medium, as described above. As a positive control chemical, EMS (CAS 62-50-0; Sigma) was used. BDO, BDO_2, and EMS were each dissolved in DMSO (CAS 67-68-5; Sigma).

hprt Mutant Frequency and Mutant Isolation

Cytotoxicity of BDO in TK6 cells (4×10^5 cells/mL in 10 mL) was assessed following a 24-hour exposure to 0, 200, 400, 600, 800, or 1,000 µM BDO. The cytotoxicity of BDO_2 in TK6 cells (4×10^5 cells/mL in 10 mL) was assessed following exposure for 24 hours to 0, 2, 4, 6, 8, or 10 µM BDO_2. Solvent control (DMSO) and unexposed cultures of TK6 cells were included with every experiment. An exposure of 20 µM EMS for 24 hours was included in BDO experiments, and 200 µM EMS for 24 hours was included with BDO_2 experiments. After the exposure period, cells were placed in fresh medium and plated to estimate relative cell survival on the basis of cloning efficiency in 96-well microtiter plates: 2 cells/well for DMSO and unexposed cells; 8 cells/well for cells exposed to BDO, BDO_2, or EMS. Cultures were then counted and diluted daily to 4×10^5 cells/mL for 10 days to allow phenotypic expression of induced *hprt* mutations and also to estimate cytotoxicity by growth curve extrapolation to the day of exposure.

To determine the *hprt* MF, parallel experiments were set up to collect *hprt* mutants from TK6 cells exposed to BDO (400 µM × 24 hours) and BDO_2 (4 µM × 24 hours) for molecular analysis. TK6 cells were exposed to BDO or BDO_2 as described above with EMS used as a positive control chemical. After the *hprt* phenotypic expression time, cells were seeded at 40,000 cells/well in 96-well microtiter plates with 1 µg/mL 6-thioguanine (6-TG) (CAS# 154-42-7; Sigma) and at 2 cells/well without 6-TG to determine cloning efficiency values. The plates were counted for growing colonies 10 days after plating. Cloning efficiency with and without 6-TG was calculated, assuming a Poisson distribution for colony formation.

For *hprt* mutant collection, six 75-cm^2 flasks containing TK6 cells (4×10^5 cells/mL in 50 mL) were exposed either to 400 µM BDO for 24 hours or to 4 µM BDO_2 for 24 hours. After exposure, the cells were placed in fresh medium, and the 50-mL contents of each flask were divided into 10-mL cultures in 25-cm^2 flasks at 1×10^5 cells/mL, for a total of 60 separate flasks. After 8 days of phenotype expression time, cells from each flask were plated on two microtiter plates at 40,000 cells/well in 6-TG (1 µg/mL). After 10 days, one clone/plate was chosen and expanded into 10 mL of medium (using 25-cm^2 flasks) with 1 µg/mL of 6-TG. To ensure that each mutant to be analyzed was independent,

cells from each flask (0.5 to 1 × 10^6 TK6 cells/mL) were then replated on two plates at the low cell density of one cell for every three wells in 6-TG (1 µg/mL). One clone was chosen per plate and placed in 10 mL medium containing 6-TG (1 µg/mL). Following expansion to 0.5 to 1 × 10^6 TK6 cells/mL, samples from each were frozen for molecular analysis of *hprt*. One mutant clone corresponding to each one of the original 60 flasks inoculated immediately after exposure was used for molecular analysis of *hprt* mutants induced by BDO or BDO_2.

RNA Isolation and Cell Lysate Preparation for DNA Analysis

Total RNA was prepared from 5 to 10 × 10^6 cells with TRIzol™ reagent and extracted with chloroform, according to the instructions of the manufacturer (GIBCO BRL). Following isopropanol precipitation, total RNA was washed in 75% ethanol and dissolved in 200 µL RNase-free water.

For DNA analysis, 0.5 to 1 × 10^6 TK6 cells were collected by centrifugation, washed once in phosphate-buffered saline (PBS), and frozen at –80°C. Frozen-cell pellets were lysed as previously described (Fuscoe et al. 1992).

hprt-Specific DNA Primers for Polymerase Chain Reaction and DNA Sequencing

The DNA primers used in this study for polymerase chain reaction (PCR) amplification and DNA sequencing were synthesized using an ABI DNA synthesizer (Perkin Elmer/Applied Biosystems, Inc., Foster City, CA). They are listed in Steen and associates (1997a,b) or are indicated in the text.

Molecular Analysis of *hprt* Mutants

Ten microliters total RNA solution was used in a final 20-µL reverse transcriptase (RT) reaction including 200 U Moloney murine leukemia virus RT (GIBCO BRL), 40 U RNasin (Promega, Madison, WI), 200 µM deoxyribonucleoside triphosphate (dNTP) (Pharmacia, Piscataway, NJ), 200 µM RT buffer (10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), and 50 pmol of *hprt* 3′ primer *hprt* 2 (Steen et al. 1997). The RT reaction was incubated in a Perkin Elmer 9600 for 5 minutes at 25°C, 60 minutes at 37°C, 5 minutes at 99°C, and 5 minutes at 4°C. For the subsequent PCR amplification, we added 80 µL PCR-mix including 200 µM dNTP (Pharmacia), 200 µM PCR buffer (10 mM Tris HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), 5 U Taq (Perkin Elmer), and 50 pmol of each primer, *hprt* 5 and *hprt* 6 (Steen et al. 1997). The PCR parameters were set for 5 minutes at 94°C followed by 30 cycles of 1 minute denaturation at 94°C and 1 minute annealing at 60°C, with the last cycle containing a 5-minute extension at 68°C. An aliquot of each of the RT-PCR products was then analyzed on a 1% agarose gel. In samples producing a poor RT-PCR product, a nested PCR amplification using primers (5′→3′) NF $_{-19}$TAC GCC GGA CGG ATC CGT T_{-1} and NR $_{695}$AGG ACT CCA GAT GTT TCC AA_{678} (*hprt* cDNA base numbers; Jolly et al. 1983) was performed using 0.5 µL from the first reaction in a final volume of 50 µL, with cycling conditions as above.

For PCR determination of genomic alterations, 8 µL of a cell-lysate sample was used as a template in a 50-µL PCR. The genomic PCR incubation conditions were the same as for RT-PCR except for the exon 1 PCR and multiplex PCR, which required a different buffer system containing 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 5 mM β-mercaptoethanol, 6.8 mM EDTA, 67 mM Tris-HCl [pH 8.8], and 10% DMSO. The multiplex PCR amplification of *hprt* exons 1, 2, 4, and 9 was performed to classify large genomic deletions among the mutants induced by BDO and BDO_2. K-*ras* was used as a DNA template control in the lysed-cell extracts. Primer concentrations used in the multiplex reaction were: 10 pmol per reaction for K-*ras* and *hprt* exon 9; 5 pmol per reaction for *hprt* exon 2 and 4; and 25 pmol per reaction for *hprt* exon 1. The PCR cycle for the multiplex reaction was 94°C for 5 minutes and 33 cycles of 94°C for 30 seconds, 61°C for 2 minutes, and 68°C for 2 minutes, followed by 5 minutes extension at 68°C, incubated in a Perkin Elmer DNA Thermal Cycler. All primers used for genomic PCR of *hprt* are listed in Steen and colleagues (1997), Gibbs and colleagues (1990), and Fuscoe and colleagues (1992), except for *hprt* ins 2S $_{29,971}$TAC CTG TAT TCA AGT CTC TAA $TA_{29,993}$ and *hprt* ins 2A $_{32,865}$ATG ATA TTT TCA ACT TCA $GA_{32,846}$ (sequences are 5′→3′; *hprt* genomic DNA base numbers; Edwards et al. 1990), which were used in the characterization of mutants B3A1 and D9A1. The same primer pairs used for PCR amplification were used for DNA sequencing.

For DNA sequence analysis, PCR amplification products were purified on Wizard columns (Promega) and eluted in 50 µL of water (Sigma). Six microliters of each purified PCR product was used in a DNA cycle sequencing reaction using the Taq DyeDeoxy™ FS Terminator cycle sequencing kit (Perkin Elmer). For cDNA PCR, the primers used were the same as used by Steen and colleagues (1997), indicated above. Sequencing reactions were precipitated with ethanol and analyzed on a 6% denaturing gel using an Applied Biosystems 373A DNA sequencer (Perkin Elmer). The sequence data were analyzed using Factura™ and AutoAssembler™ sequence analysis software (Perkin Elmer). Mutations were confirmed by visual inspection of each histogram and comparison with a wild-type sequence.

Statistical Comparison of the BDO and BDO_2 Mutational Spectra to Background Mutations in Human TK6 Cells

The frequency and percentage of all mutational types among the BDO- and BDO_2-induced mutants were compiled

for statistical analysis. The mutational spectrum determined for a collection of spontaneous mutants isolated from the same TK6 stock cultures (Steen et al. 1997a) was compared with the BDO and BDO_2 mutational spectra to identify BDO- or BDO_2-specific mutations. The MFs from background, BDO, and BDO_2 groups were used to calculate the contribution of each mutational class to the *hprt* mutation frequency. Because only increases were expected in the mutation frequencies analyzed, Fisher exact test (one-tailed; $p < 0.05$) was used to test for significant increases between groups, using the mutation frequency calculated for each particular genotypic change. All statistical analyses were carried out with JMP® statistical analysis software (SAS Institute, Cary, NC).

SPECIFIC AIM 2. THE IN VITRO MUTAGENICITY AND MUTATIONAL SPECTRA OF BDO AND BDO_2 AT THE *lacI* TRANSGENE IN Rat2 CELLS

Chemicals

Chemicals (and sources) used in this specific aim are: *N*-ethyl-*N*-nitrosourea (ENU) (Sigma), DMSO (Aldrich, Milwaukee, WI), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Gold Biotechnologies, St. Louis, MO), and dimethylformamide (Sigma).

Rat2 *lacI* Transgenic (Big Blue™) Fibroblasts

The Rat2 cells (Stratagene, La Jolla, CA) used in these experiments were constructed by cotransfection of the rat embryonic cell line Rat2 with the lambda *lacI* shuttle vector and the pSV2NEO plasmid (Wyborski et al. 1995). The lambda *lacI* shuttle vector, which has integrated into the Rat2 genome at two sites (approximately 60 copies per cell), is identical to the one used to generate the Big Blue™ *lacI* transgenic mouse.

Cell Culture and Exposures

The Rat2 cells were cultured as a monolayer in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL) containing 2 mM L-glutamine, 60 IU/mL penicillin, 60 mg/mL streptomycin, 200 µg/mL geneticin (G418, Gibco BRL), and 10% heat-inactivated defined fetal bovine serum (HyClone) at 37°C in a humidified incubator (Forma Scientific) containing 10% CO_2. Under these conditions, the average cell cycle time was approximately 19 hours. Low passage (< 5) cells were used in each experiment. Cells were treated with BDO or BDO_2 at 30% to 40% confluence (approximately 2.5×10^6 cells in a 75-cm^2 flask). A solution of 0.25% trypsin (Gibco BRL) was used to detach cells for subculture.

Cytotoxicity of BDO in Rat2 Cells

The survival of Rat2 cells following exposure to BDO was assessed by exposing two sealed 75-cm^2 flasks (each containing approximately 2.5×10^6 cells) for each of five levels of concentration (0, 0.4, 0.6, 0.8, or 1.2 mM BDO) for 24 hours. Cells from each exposed flask were then trypsinized and counted with a Coulter Counter® ZM (Coulter Instruments), and replicate flasks were plated with 50,000 cells each, using 150-cm^2 flasks. For each exposed flask there were four replicate flasks to be counted at 24, 72, 120, and 168 hours after the initial plating. Survival at each exposure concentration was assessed in terms of cumulative cell growth relative to media controls at 120 hours after plating.

Mutagenicity of BDO in Rat2 Cells

The mutagenicity of BDO at *lacI* in Rat2 cells was assessed in two independent experiments by exposing two or three sealed 75-cm^2 flasks (each containing approximately 2.5×10^6 cells) to BDO concentrations of 0, 0.6, or 1.0 mM BDO for 24 hours. (Two replicates were used per concentration in the first experiment; three replicates were used per concentration in the second.) Solvent controls (0.1% DMSO) and positive controls (10 mM ENU) were included in both experiments. The two experiments were carried out identically with respect to exposure of the cells to BDO and the assessment of mutagenicity. Following the 24-hour exposure, cells from each exposed flask were trypsinized and counted with a Coulter Counter® ZM, and one 150-cm^2 flask per exposed flask was plated with 800,000 cells. When cells had expanded to confluence, they were trypsinized, pelleted, and flash frozen in liquid nitrogen. The time required for cells to reach confluence varied with BDO exposure. Cells in all flasks were confluent by the fourth day after plating except for those exposed to 1.0 mM BDO, which were confluent by the fifth day. Cell pellets were stored at –80°C. Each pellet, representing a single exposed flask, was considered the experimental unit.

Determination of *lacI* Mutant Frequency in Rat2 Cells Exposed to BDO

Mutant frequency was assessed in cells exposed to 0.6 or 1.0 mM BDO and assessed also in the media, solvent, and positive controls from the two experiments. Genomic DNA was prepared from cell pellets using the Megapore™ method (Stanford Research Institute, International, Menlo Park, CA). Briefly, cell pellets were thawed on ice and resuspended in 5 mL of an ice-cold cell lysis solution (10 mM Tris HCl [pH 8.3], 140 mM NaCl, 3 mM KCl , 0.35 M sucrose, 1 mM EDTA, and 1% Triton X-100) for 5 minutes. These suspensions were filtered through a 100-µm nylon mesh filter (Tetko Inc., Briarcliff Manor, NY)

into 50-mL tubes, which were centrifuged at 1,100 × *g* at 4°C for 12 minutes. The supernatant was removed and the pellet was rinsed with 3 mL cell lysis solution, and the centrifugation was repeated for 5 minutes. The supernatant was then removed, and tubes were wiped with a cotton swab to remove residual supernatant. The pellets were resuspended in 7.5 mL of tissue homogenization buffer (10 mM Tris HCl; 150 mM NaCl , pH 8.0; from Stratagene), and an equal volume of a proteinase K solution (2 mg/mL proteinase K [Sigma], 2% sodium dodecyl sulfate [SDS], 100 mM EDTA) was added. The solutions were incubated at 50°C for 15 minutes and then transferred onto a 0.45-µm filter (Millipore), which was floating in a 100-mm Petri dish containing 35 mL of TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 7.5). After 18 to 20 hours, the DNA was removed from the filters and stored at 4°C.

The lambda shuttle vector containing the *lacI* transgene was recovered from genomic DNA with lambda-phage packaging extract (Transpack, Stratagene), according to manufacturer's instructions. Packaged phage were preadsorbed to *Escherichia coli* SCS-8 cells for 20 minutes at 37°C, mixed with prewarmed NZY top agar containing 1.3 mg/mL X-gal (dissolved in dimethylformamide), and poured into 22.3-cm^2 assay trays (Vanguard International, Neptune NJ) containing NZY agar. Plates were incubated at 37°C overnight.

The total number of clear *lacI*$^+$ plaques was estimated for each plate by counting three 6.25-cm^2 sections, finding their average, and multiplying by a scaling factor. Blue *lacI*$^-$ plaques were counted and placed into individual tubes (containing 0.5 mL SM buffer (50 mM Tris HCl, pH 7.5; 10 mM $MgSO_4$; 0.001% gelatin; 0.1 M NaCl) and 50 µL chloroform) for further characterization. All mutant plaques were confirmed by restreaking on an SCS-8 bacterial lawn growing on NZY top agar containing X-gal.

The *lacI* MF was calculated for each sample by dividing the number of confirmed mutant plaques by the total number of plaques analyzed. For each sample, more than 200,000 plaques were analyzed.

DNA Sequence Analysis of *lacI* Background Mutants and Mutants Induced by BDO

The *lacI* gene was sequenced from *lacI* mutants isolated from solvent and media control cells (collectively considered as controls) and from cells exposed to 1.0 mM BDO. Mutants to be sequenced were selected from the two mutagenicity experiments such that they were distributed evenly among all of the independently exposed samples. Each mutant was purified by replating at low density on a 100-mm Petri dish. At this time, plaques were scored for blue color intensity. From each dish, a single *lacI*$^-$ plaque was scored and placed into 50 µL distilled water and boiled for 5 minutes. Following centrifugation, the *lacI* gene was amplified using 2 µL of the supernatant from the boiled plaque as the DNA template in a PCR. The PCR products were sequenced directly, using a Taq DyeDeoxy™ Terminator sequencing kit on an Applied Biosystems 373 DNA sequencer (Perkin Elmer). Sequence data were analyzed with Factura™ and AutoAssembler™ software (Perkin Elmer). Each *lacI* mutant was sequenced in its entirety, and all mutations were determined by at least two primer reactions. The first G in the GTG start codon is base number 29 (Farabaugh et al. 1978). Mutants were considered to be siblings if the same mutation occurred more than once in an independent Rat2 cell sample.

Statistical Analysis of BDO Mutational Spectrum Compared with Background Mutations

The frequency and percentage of all mutational types among the BDO-induced and background Rat2 cell mutants were compiled for statistical analysis. The mutational spectrum determined for a collection of spontaneous mutants was compared to the BDO mutational spectrum to identify BDO-specific mutations. To examine the mutational spectrum data for statistical differences among the specific mutational types determined for the BDO-induced mutants compared to background mutants, the mutation frequency for each group was calculated as described in Carr and Gorelick (1996). Since only increases were expected in the mutation frequencies analyzed, a one-way Fisher exact test ($\alpha = 0.05$) was used to test for significant differences between the groups, using the mutation frequency calculated for each particular genotypic change. All statistical analyses were carried out with JMP® statistical analysis software (SAS Institute, Cary, NC).

Cytotoxicity of BDO_2 in Rat2 Cells

Relative percent survival of Rat2 cells following exposure to BDO_2 was determined by assessing relative cumulative growth at 120 hours after exposure. Three replicates per exposure concentration were exposed for 24 hours to 0, 2, 5, or 10 µM BDO_2 (Sigma) dissolved in DMSO. Cells were then returned to normal culture conditions, and cumulative cell growth was assessed at 24, 72, 120, and 168 hours.

Determination of *lacI* Mutant Frequency in Rat2 Cells Exposed to BDO_2

The mutagenicity of BDO_2 at *lacI* in Rat2 cells was determined in three independent experiments by exposing at least three 75-cm^2 flasks per exposure concentration to 0, 2, 5, or 10 µM BDO_2 for 24 hours. Solvent controls (0.1% DMSO) were also included. The 2, 5, and 10 µM concentrations correspond to approximately 100%, 50%, and

10% relative survival, respectively. As a positive control, 10 mM ENU was used in all experiments. Following the 24-hour exposure, cells were returned to normal culture conditions, then subcultured to 2.5×10^6 cells when they reached confluence. Cells were collected and flash frozen in liquid nitrogen 72 hours postexposure. DNA was then isolated, and the MF was determined as described (Sisk et al. 1994).

Cytokinesis-Block Micronucleus Assay in Rat2 Cells

In two independent experiments, two to four cultures of Rat2 cells per exposure concentration in 75-cm^2 flasks were exposed to 0, 2, 5, or 10 µM BDO_2. Cytochalasin B was added at 1 µg/mL to block cytokinesis and to induce the accumulation of binucleated cells (Saranko and Recio 1998). In the second experiment, solvent controls (0.1% DMSO) and a 0.5 µM BDO_2 exposure concentration were also included. Cells were incubated for 24 hours at 37°C before harvesting.

Following the 24-hour exposure, the cultures were rinsed with warm Dulbecco's PBS (Gibco BRL) and detached with 0.25% trypsin. One flask of cells per exposure concentration was counted with a Coulter Counter® ZM (Coulter Instruments). Based on cell number, the other flasks representing each exposure concentration were resuspended in DMEM containing 15% fetal bovine serum at 2.5×10^5 cells/mL. Approximately 0.2 mL of the cell suspension was centrifuged onto clean slides at 500 rpm for 4 minutes using a cytocentrifuge (Cytospin®, Shandon). The slides were briefly air dried and then fixed in 100% methanol at –20°C for 15 minutes. Following fixation, slides were stored with desiccant under a nitrogen atmosphere at –20°C.

Before being scored for micronuclei, slides were randomized and stained with a 0.02% solution of acridine orange (Sigma) in PBS for 10 minutes at 4°C. Slides were rinsed in 3 changes of PBS for 3 minutes each. Cover slips were mounted with 1 drop of PBS immediately and then scored within 48 hours.

Scoring of Micronuclei in Rat2 Cells

Slides were scored for micronuclei using a Nikon Microphot-FXA microscope fitted with fluorescence equipment and a B-2A filter. Criteria based on those of Lynch and Parry (1993) were followed: (1) Micronuclei frequencies were determined in 1,000 binucleated cells per replicate. (2) Binucleated cells with any number of micronuclei were counted as a single event. (3) Micronuclei were counted only if the cell was intact and had two parent nuclei; only micronuclei with diameters smaller than 50% of the parent nuclei were counted. (4) Only micronuclei distinctly separate from the parent nuclei were counted. A nuclear division index for each exposure treatment was calculated as follows, counting the number of nuclei in at least 400 cells per replicate: Nuclear division index = $[(M1) + (2 \times M2) + (3 \times M3) + (4 \times M4)]/N$, where $M1$ to $M4$ represent the cells with 1 to 4 nuclei, and N is the total number of cells scored (Eastmond and Tucker 1989).

Statistical Analysis of *lacI* Mutant Frequency in Rat2 Cells Exposed to BDO or BDO_2 and Micronuclei Frequency in Rat2 Cells Exposed to BDO_2

Using methods recommended by Callahan and Short (1995), the *lacI* MF was log-transformed and tested for significant differences (air-exposed controls compared to BDO- or BDO_2-exposed groups), using Student t test with significance levels of $p < 0.05$. In addition, analysis of variance (ANOVA) was used to test for differences between treatment groups in the mutagenicity and micronuclei experiments. In both cases, a Dunnett multiple-comparison test (Miller 1980) was used to compare each treatment group to its corresponding control. A linear regression analysis was performed on the data from the BDO_2 mutagenicity experiments to examine for increasing trends between treatment and response (i.e., MF). All statistical analyses were carried out with JMP® statistical analysis software (SAS Institute, Cary, NC).

SPECIFIC AIM 3. THE IN VIVO MUTAGENICITY OF BDO AND BDO_2 IN $B6C3F_1$ *lacI* TRANSGENIC MICE AND F344 *lacI* TRANSGENIC RATS

Chemicals

A racemic mixture of BDO was purchased from Aldrich (St. Louis, MO) and found to be 97.4% pure by nuclear magnetic resonance (NMR) analysis. A racemic mixture of BDO_2 was purchased in lots from Aldrich and from Lancaster (Windham, NH), which were combined and redistilled to obtain purity of 99.2% by NMR analysis.

lacI Transgenic Mice and Rats

Female $B6C3F_1$ *lacI* mice and male and female F344 *lacI* rats were purchased from Stratagene Cloning Systems, Inc. (Taconic Farms, Germantown, NY). All animals were 4 to 6 weeks of age. They were acclimatized for at least 10 days prior to experimental exposure and were weighed once weekly throughout the in vivo part of the study. Prior to exposures, animals were randomized by weight for assignment to exposure groups.

Since female mice and rats are more sensitive to the carcinogenic effects of BD than male mice and rats, females were chosen for the present studies. However, because sufficient numbers of female rats were not available at the time of BDO_2

exposure, male rats were added to ensure at least eight animals per exposure group (four females and four males in the BDO_2-exposed groups) (Callahan and Short 1995).

Inhalation Exposures to BDO and BDO_2

Inhalation exposures of BDO and BDO_2 were conducted at the Lovelace Respiratory Research Institute (Albuquerque, NM) under the direction of Dr. Rogene F. Henderson. Rats and mice were exposed to target concentrations of 25 ppm BDO or 4 ppm BDO_2 for 6 hours/day, 5 days/week, for 2 weeks. Air-exposed controls (0 ppm) were included with each chemical exposure. The actual exposure (mean ± SD) concentrations were 29.9 ± 1.8 ppm for BDO and 3.8 ± 0.6 ppm for BDO_2.

Four multitiered whole-body exposure chambers (H-2000, Lab Products, Inc., Aberdeen, MD) were used in the exposures. The total volume of the H-2000 chamber is 1.7 m^3. Flow rate through each chamber was maintained at 15 ± 2 ft^3/min, which corresponds to 15 ± 2 air changes per hour. All air was HEPA-filtered before being introduced into the chamber supply system. Vapors of BDO and BDO_2 were generated by using a J-tube generation system. Control animals in one chamber received only filtered air, whereas rodents in the other chambers received high or low concentrations of test compounds. Each daily exposure was 6 hours plus the time needed for the exposure chamber to reach 90% of the target concentration (T_{90}); T_{90} values for each level were determined before the start of exposures. The animals were housed in exposure chambers throughout the experiment, and had free access to food (NIH-07 certified feed) and water except during the 6-hour exposure times, when food was removed.

In the J-tube generation system, BDO_2 or BDO was injected into the glass J-tube by a syringe pump. The feed rate was varied to control chamber concentration. The Miran IA infrared spectrometer (Foxboro, Inc., Foxboro, MA) was used periodically to monitor chamber vapor concentrations of BDO and BDO_2 in real time. The Miran is a single-beam, variable-filter infrared spectrometer. The gas cell parameters (volume, slit, response time), wavelength, and path length were adjusted for optimum operation for each compound. The BDO atmospheres were quantified by gas chromatographic analysis of grab samples. To quantify the BDO_2 atmospheres, bubbler samples were extracted three times daily from each chamber. Sample trains consisted of two glass impingers connected in series, with each impinger containing 50 mL of ethyl acetate. Sample trains were kept on ice and operated at 2 L/min for 1 hour. The bubbler sample was then analyzed by gas chromatography/mass spectroscopy.

In Vivo Mutagenicity Experiments in *lacI* Transgenic Mice and Rats

To assess the in vivo mutagenicity of BDO, animals were exposed to 0 or 29.9 ppm BDO for 6 hours/day, 5 days/week, for 2 weeks. This exposure level was chosen to achieve BDO blood levels (3.7 µM BDO) similar to those in pharmacokinetic models (Medinsky et al. 1994) reported for mice exposed once for 6 hours to 625 ppm of the parent compound BD. Each group numbered 8 female mice or 8 female rats, as recommended by Callahan and Short (1995).

To assess the in vivo mutagenicity of BDO_2, animals were exposed to 0 or 3.8 ppm BDO_2 for 6 hours/day, 5 days/week, for 2 weeks. Preliminary experiments indicated that 3.8 ppm BDO_2 would not induce significant mortality over the 2-week exposure period. This exposure level was predicted to result in blood levels of BDO_2 similar to those in pharmacokinetic models (Medinsky et al. 1994) reported for mice exposed once for 6 hours to 62.5 ppm of the parent compound BD (0.005 µM to 0.65 µM BDO_2). The mouse BDO_2 exposure and control groups consisted of six females per group, whereas the rat BDO_2 exposure and control groups consisted of four females and four males per group.

Animals were exposed to each metabolite for 2 weeks followed by a 2-week postexposure sampling time for bone marrow in mice and rats, a 2-week sampling time for spleen in mice, and a 5-week sampling time for *lacI* mutants in the spleen of rats. These fixation times were based on studies in *lacI* mice exposed to BD (Sisk et al. 1994; Recio et al. 1996, 1997) and studies at *hprt* in splenocytes from rats exposed to BD (Meng et al. 1999).

Animals were killed by CO_2 asphyxiation and cardiac exsanguination. Tissues sampled for each animal included lung, liver, spleen, bone marrow, and kidney. Excised samples were placed immediately in appropriate containers, flash frozen in liquid nitrogen, then stored frozen at –80°C. Bone marrow cells were collected by flushing marrow from both femurs with PBS into 2-mL microcentrifuge tubes. The cell suspensions were centrifuged for 30 seconds at maximum speed. The supernatant was discarded, and the pellets were flash frozen in liquid nitrogen and then stored at –80°C.

Determination of the *lacI* Mutant Frequency in Bone Marrow and Spleen Cells of *lacI* Transgenic Mice and Rats Exposed to BDO or BDO_2

DNA was isolated from bone marrow and spleen using essentially the same methods as used for Rat2 cells. Briefly, bone marrow–cell pellets or excised portions of spleen were thawed on ice in 5 mL of an ice-cold cell lysis solution, and DNA was isolated as described above. The *lacI* MF was determined using the same methods used to assess the in vitro mutagenicity of BDO and BDO_2 in Rat2 cells.

DNA was isolated from lung samples according to Stratagene's *Big Blue™ Transgenic Mouse Assay System Instruction Manual*. Following ethanol precipitation, the DNA was resuspended in 200 µL TE buffer and kept at room temperature overnight before storing at 4°C.

Statistical Analysis of *lacI* Mutant Frequency in Mice Exposed to BDO or BDO_2

Using methods recommended by Callahan and Short (1995), the *lacI* MF was log-transformed and tested for significant differences (air-exposed controls compared to BDO- or BDO_2-exposed groups) using Student *t* test, with significance levels of $p < 0.05$.

RESULTS

SPECIFIC AIM 1. THE IN VITRO MUTAGENICITY AND MUTATIONAL SPECTRA OF BDO AND BDO_2 AT *hprt* IN HUMAN TK6 CELLS

Cell Survival and *hprt* Mutant Frequency in Human TK6 Cells Exposed to BDO

Cytotoxicity of BDO was assessed in TK6 cells (4×10^5 cells/mL in 10 mL) following a 24-hour exposure to 0, 200, 400, 600, 800, or 1,000 µM BDO using cloning efficiency and growth curve extrapolation. Findings of a 7% relative survival after an exposure to 400 µM BDO (Steen et al. 1997b) were consistent with data reported previously (Cochrane and Skopek 1994a). In the present study, the 400 µM BDO exposure used to generate *hprt* mutants for molecular analysis induced a five- to ninefold increase in the *hprt* MF (in two different cultures) above the spontaneous *hprt* MF (Table 1).

Table 1. Mutagenicity of BDO and BDO_2 at the *hprt* Locus in Human TK6 Cells

	hprt Mutant Frequency ($\times 10^{-6}$)[b]	
Exposure[a]	BDO Exposure	BDO_2 Exposure
Control	4.4 ± 1.1	3.2 ± 0.8
DMSO (0.1%)	3.9 ± 0.1	2.6 ± 0.3
BDO (400 µM)	30.1 ± 7.3	
BDO_2 (4 µM)		17.0 ± 6.0
EMS (20 µM)	16.3 ± 0.1	
EMS (200 µM)		223.0 ± 1.0

[a] All exposures were for 24 hours.

[b] Values are means ± SD.

Molecular Analysis of *hprt* Mutants Isolated from Human TK6 Cells Exposed to BDO

All BDO-induced mutants were initially analyzed by *hprt* cDNA-specific RT-PCR and agarose gel electrophoresis. *hprt*-Specific RT-PCR–amplified products were obtained from 47 of 49 (96%) of the mutants isolated. Two mutants, A9A1 and E7A1, did not produce an RT-PCR product and were then analyzed for genomic deletions by multiplex PCR of *hprt* exons 1, 2, 4, and 9, using K-*ras* as a DNA template control. Both A9A1 and E7A1 produced a PCR product for K-*ras* but not for the *hprt* exons tested and were thus classified as having total *hprt* deletions.

Of 49 BDO-induced mutants analyzed, 39 (78%) had point mutations, and 11 (22%) had deletions that ranged in size from single deletions to large deletions of *hprt* (Table 2). In the 49 mutants analyzed, we observed 50 mutations because one mutant, D3A1, had two point mutations (Table 2). Point mutations observed in 39 mutants were evenly distributed between G•C base pairs (18 of 39) and A•T base pairs (21 of 39) and between the proportion of transitions (21 of 39) and transversions (18 of 39). In three mutants, E5A1, B1A1, and F10A1, a single base-pair deletion resulted in frameshift mutations in the *hprt* cDNA (Table 2).

hprt RT-PCR products with internal exon(s) missing were obtained in 21 of 49 BDO-induced mutants. Genomic PCR of the corresponding missing exon(s) in the *hprt* cDNA resulted in products for 18 of these 21 mutants. On DNA sequence analysis of the PCR-amplified products from genomic DNA, 16 of 21 mutants had point mutations in the splice acceptor or donor sites, which were probably responsible for the aberrant splicing. Mutant D3A1, a mutant with exon 8 skipping in the *hprt* cDNA, had 2 point mutations within exon 8. Mutant D1A1 had a 39-bp deletion extending 21-bp into exon 6 that resulted in the splicing out of exon 6 from *hprt* mRNA. Genomic deletions of entire exons were found in three mutants (B9A1, B10A1, and F7A2) that produced short *hprt* RT-PCR products (Table 2).

The BDO mutational spectrum obtained in the present study was summarized and compared to the background mutational spectrum (Steen et al. 1997a) determined from the same TK6 stock cell cultures (Table 3). There was a significant increase in G•C→A•T transitions (one-tailed Fisher exact test; $p < 0.05$), A•T→G•C transitions ($p = 0.0001$), and A•T→T•A transversions ($p < 0.0001$) among the BDO-induced mutants compared to background mutants (Steen et al. 1997). Most notable were the A•T→T•A transversions, which were found in 24% of the BDO-induced mutants compared to 5% of background mutants. In addition to this fourfold increase, the A•T→T•A transversion mutation frequency increased 19-fold

Table 2. DNA Sequence Analysis of *hprt* Mutations Isolated from Human TK6 Cells[a]

Mutant	Base Pair Number[b]	DNA Sequence Context[c] 5′→3′	Mutant Codon	Base Change	Predicted Amino Acid Change	Exon Affected
Base-Substitution Mutations						
A7B1	3	GTT ATG GCG	ATA	G→A	Met→Ile	1
D6A1	209	AAG GGG GGC	GAG	G→A	Gly→Glu	3
A2A1	255	GCA CTG AAT	CTC	G→C	Leu→Leu	3
E2A1	325	GAC CAG TCA	TAG	C→T	Gln→term	4
C2A1	400	GTG GAA GAT	AAA	G→A	Glu→Lys	5
B6A1	454	AGG CAG TAT	TAG	C→T	Gln→term	6
B5A1	538	GTT GGA TTT	AGA	G→A	Gly→Arg	8
C4A1	538	GTT GGA TTT	AGA	G→A	Gly→Arg	8
E9A1	539	GTT GGA TTT	GAA	G→A	Gly→Glu	8
C3B1	568	GTA GGA TAT	AGA	G→A	Gly→Arg	8
E6A1	580	CTT GAC TAT	AAC	G→A	Asp→Asn	8
F3A1	IN3:1 (16,787)	TAT TGT] gtg act	atg	G→A	In-frame deletion	2, 3[d]
F9A1	IN3:1 (16,787)	TAT TGT] gtg act	atg	G→A	Frameshift[e]	3[d]
D8A2	IN4:1 (27,958)	GGG AAG] gta tgt	ata	G→A	In-frame deletion	4[d]
A8A1	IN4:-1 (31,617)	ttc tag [AAT GTC	tat	G→T	In-frame deletion	5[d]
C10B1	IN4:-1 (31,617)	ttc tag [AAT GTC	tac	G→C	In-frame deletion	5[d]
A10A1	IN6:1 (35,021)	CGC AAG] gta tgt	ata	G→A	Frameshift	6[d]
D3A1	539	TTG GAT TTG	TAT	G→T	Frameshift	8[d]
E1A1	124	CTA ATT ATG	TTT	A→T	Ile→Phe	2
F4A1	215	GGC TAT AAA	TTT	A→T	Tyr→Cys	3
C5A1	301	ATC AGA CTG	TGA	A→T	Arg→term	3
C6A1	301	ATC AGA CTG	TGA	A→T	Arg→term	3
D10A1	343	ATA AAA GTA	TAA	A→T	Lys→term	4
B7A1	374	ACT TTA ACT	TGA	T→G	Leu→term	4
D4A1	404	GAA GAT ATA	GTT	A→T	Asp→Val	6
C9B1	523	TAT AAG CCA	TAG	A→T	Lys→term	7
C1A1	611	AAT CAT GTT	CTT	A→T	His→Leu	9
C7A1	618	GTT TGT GTC	TGG	T→G	Cys→Trp	9
B4A1	IN2:-2 (16,601)	ctg tag [GAC TGA	tag	A→G	In-frame deletion	2, 3[d]
D5A1	IN2:-2 (16,601)	ctg tag [GAC TGA	ttg	A→T	In-frame deletion	2, 3[d]
D7A1	IN2:-2 (16,601)	ctg tag [GAC TGA	tgg	A→G	Frameshift	3[d]
F8A1	IN2:-2 (16,601)	ctg tag [GAC TGA	tgg	A→G	Frameshift	3[d]
B2A1	IN4:-2 (31,616)	ttc tag [AAT GTC	tgg	A→G	In-frame deletion	5[d]
D2A1	IN4:-2 (31,616)	ttc tag [AAT GTC	tgg	A→G	In-frame deletion	5[d]
F6B2	IN7:3 (39,865)	CAG ACT] gta agt	gtt	A→T	Frameshift	7[d]
E4A1	IN8:4 (40,114)	TTG AAT] gta agt	tgt	A→T	Frameshift	8[d]
F5A1	IN8:4 (40,114)	TTG AAT] gta agt	tgt	A→T	Frameshift	8[d]
D3A1	552	ATT CCA GAC	CCT	A→T	Frameshift	8[d]
E10A1	IN8:-2 (41,453)	tta tag [CAT GTT	tgg	A→G	Frameshift	8[d]

(*Table continues next page*)

[a] TK6 cells were exposed to 400 μM BDO for 24 hours.

[b] For *hprt* cDNA, base number 1 is the A in the AUG start codon. For genomic DNA, designation such as IN3:1 refers to the first base of intron 3, and IN4:1 refers to the last base of intron 4. The numbering system for the entire gene (Edwards et al. 1990) is given in parentheses.

[c] Upper-case letters are exon sequence, and lower-case letters are intron sequence.

[d] Exon skipped at the cDNA level.

[e] In mammalian cells, a base-substitution mutation in an intron sequence can result in aberrant splicing of mRNA (exon skipping) that will result in a frameshift in the amino acid codons for *hprt*.

Table 2. DNA Sequence Analysis of *hprt* Mutations Isolated from Human TK6 Cells[a] (*Continued*)

Mutant	Base Pair Number[b]	DNA Sequence Context[c] 5′→3′	Mutant Codon	Base Change	Predicted Amino Acid Change	Exon Affected
Deletions						
E5A1	154	CGA GAT GTG		Del G	Frameshift	3
B1A1	640	AAA GCA AAA		Del G	Frameshift	9
F10A1	648			Del C	Frameshift	9
B3A1	IN: (30,146–32,825)			Del 2,680 bp	Deletion	
D9A1	IN: (30,146–32,825)			Del 2,680 bp	Deletion/insertion	
D1A1	IN: (34,920–34,958)			Del 39 bp	Frameshift	
B9A1	Genomic deletion			– Exon 2	Frameshift	2
B10A1	Genomic deletion			– Exon 4	In-frame deletion	4
F7A2	Genomic deletion			– Exon 6	Frameshift	6
A9A1	Total deletion					
E7A1	Total deletion					

[a] TK6 cells were exposed to 400 μM BDO for 24 hours.

[b] For *hprt* cDNA, base number 1 is the A in the AUG start codon. For genomic DNA, designation such as IN3:1 refers to the first base of intron 3, and IN4:1 refers to the last base of intron 4. The numbering system for the entire gene (Edwards et al. 1990) is given in parentheses.

[c] Upper-case letters are exon sequence, and lower-case letters are intron sequence.

[d] Exon skipped at the cDNA level.

[e] In mammalian cells, a base-substitution mutation in an intron sequence can result in aberrant splicing of mRNA (exon skipping) that will result in a frameshift in the amino acid codons for *hprt*.

Table 3. A Summary of the *hprt* Mutational Spectra for Background, BDO-, and BDO_2-Induced Mutations in Human TK6 Cells[a]

Mutation Type	Background[b]	BDO-Induced	BDO_2-Induced
Base substitutions			
Transitions			
A•T→ G•C	3 (7%)	6 (12%)	1 (2%)
G•C→ A•T	10 (23%)	14 (28%)	3 (6%)
Transversions			
A•T→ C•G	3 (7%)	2 (4%)	1 (2%)
A•T→ T•A	2 (5%)	12 (24%)[c]	9 (18%)
G•A→ C•G	1 (2%)	2 (4%)	2 (4%)
G•C→ T•A	3 (7%)	2 (4%)	2 (4%)
Other alterations			
Insertions	0 (< 2%)	1 (2%)	1 (2%)
Genomic *hprt* deletions (total)	21 (49%)	11 (22%)[c]	32 (63%)
Partial 5′ deletions	1 (2%)	0 (< 2%)	7 (14%)[c]
Partial 3′ deletions	9 (21%)	0 (< 2%)	7 (14%)
Internal	9 (21%)	9 (18%)	13 (25%)
Total deletions	2 (5%)	2 (4%)	5 (10%)
Total mutations analyzed	43	50	51

[a] TK6 cells were exposed for 24 hours to a racemic mixture of 400 μM BDO or 4 μM BDO_2.

[b] Data are from Steen and associates (1997a).

[c] Significant increases compared with background mutations (one-tailed Fisher exact test).

in BDO-exposed TK6 cells (3.04×10^{-6}) compared to the background mutation frequency (0.16×10^{-6}) (Steen et al. 1997).

Examination of the DNA sequence context of the G•C→A•T transitions and A•T→T•A transversions determined among the BDO-induced mutants demonstrated that there was strand bias in the induction of mutations. The purine base (either guanine or adenine) occurred frequently on the *hprt* nontranscribed strand. Among the 14 G•C→A•T transitions, the G was located in the nontranscribed strand in 12 of 14 (86%). In 19 of 21 (90%) of the mutations observed at A•T base pairs, the A was located in the nontranscribed strand. All of the A•T→T•A transversions occurred with the A in the nontranscribed strand.

Cell Survival and *hprt* Mutant Frequency in Human TK6 Cells Exposed to BDO_2

Two experiments were done to determine the relative percentage of survival and *hprt* MF in TK6 cells (4×10^5 cells/mL in 10 mL) exposed for 24 hours to 0, 2, 4, 6, 8, or 10 μM BDO_2. Cytotoxicity was assessed by cloning efficiency and growth curve extrapolation. Both methods indicated that 4 μM BDO_2 for 24 hours results in approximately 10% relative survival (Steen et al. 1997a). These data are consistent with those reported by Cochrane and Skopek (1994a). In their mutagenicity dose-response experiments in TK6 cells (1994a), the maximal *hprt* mutagenic response induced by BDO_2 occurred at exposures of 3.9 μM BDO_2 for 24 hours. Therefore, in the present study, exposures of 4 μM BDO_2 for 24 hours were used to conduct a mutagenicity experiment and to initiate cultures for *hprt* mutant isolation from BDO_2-exposed TK6 cells. In the mutagenicity experiment, 50-mL cultures of TK6 cells at 4×10^5 cells/mL were exposed to 4 μM BDO_2 for 24 hours, resulting, on average, in a fivefold increase in MF above background (Table 1).

Molecular Analysis of *hprt* Mutants Isolated from Human TK6 Cells Exposed to BDO_2

All BDO_2-induced mutants were initially analyzed by *hprt* cDNA-specific RT-PCR and agarose gel electrophoresis. Of 51 *hprt* mutants isolated from BDO_2-exposed TK6 cells, 32 resulted in an RT-PCR product visible on a 1% agarose gel. More than half these 32 mutants had an altered migration pattern on the agarose gel, suggesting exon loss or genomic deletion.

Of the 51 mutants, 19 produced no RT-PCR product and were further analyzed by exon-specific PCR amplification of exons 1, 2, 4, and 9 to assess genomic *hprt* for exon loss (Table 4). Of the 19, 5 produced no exon-specific PCR products and were considered to have large deletions; 7 produced no PCR amplification product from exon 1, exons 1 and 2, or exons 1, 2, and 4, and were considered to be partial 5′ deletion mutants; 7 produced no PCR product for exon 9, or exons 4 and 9, and were considered to be partial 3′ deletion mutants.

Mutants with an altered cDNA length were the result of exon losses (exon skipping) in 20 of 51 BDO_2-induced mutants. Of the 20, 13 mutants showed single exon loss; 5 demonstrated exon loss for two consecutive exons; and 2 showed exon loss that included three exons, 1 with 4, 5, and 6 exon skipping, and 1 with 6, 7, and 8 exon skipping (Table 5). These 20 mutants were examined at the genomic level by exon-specific PCR to determine the mutational basis for the altered *hprt* cDNA products. Intron primers that would produce a PCR product encompassing the exon(s) absent in the cDNA (and flanking regions) were used to PCR-amplify the corresponding genomic regions of *hprt*. Certain PCR products (e.g., mutants with single exon skipping in cDNA) were analyzed by DNA sequencing and examined for mutations in splice donor or acceptor sites that could result in aberrant splicing of *hprt* mRNA. Of the 13 BDO_2-induced mutants that showed single exon loss, 6 were due to single base-pair substitutions in splice-site sequences (Table 6). Mutant 3B42A had a T→A transversion in the intron 1 donor splice site resulting in the inclusion of 48 bp of intron 1 sequence between exon 1 and 2 in *hprt* cDNA. Mutant 3A31A had a 1-bp deletion in the donor site (Table 5) resulting in exon 8 skipping, whereas mutant 3A51B, also with exon 8 skipping, had a C→T mutation within exon 8 (Table 6). Mutant 3F32A had a 46-bp deletion within exon 6 that resulted in exon 6 skipping in *hprt* cDNA. The remaining BDO_2-induced mutants with single exon skipping had either genomic deletions of the corresponding exon sequences in the genomic DNA or mutations affecting PCR primer annealing sites. Two BDO_2-induced mutants with exons 2 and 3 absent in the *hprt* cDNA (3D11B and 3F92A) had genomic deletions of exon 3. The remaining mutants with either 2 or 3 exons absent in *hprt* cDNA had the corresponding regions deleted from genomic *hprt*. These mutants were not further characterized to assess breakpoint junctions.

One BDO_2 mutant (3C91B) had a 4-bp insertion of an ATTG in exon 4; that is, a duplication of the preceding 4 bases (Table 5). Mutant 3A31A had a single base-pair deletion in the splice donor region resulting in exon 8 skipping in *hprt* cDNA.

Single base-pair substitutions in the *hprt* coding region were identified in 10 of 51 BDO_2-induced mutants, of which 8 were transversions and 2 were transitions (Tables 3 and 6). Single base-pair substitutions were found at intron splice site sequences in 6 mutants that resulted in aberrant splicing of *hprt* mRNA (by exon skipping or

Table 4. Results of Exon-Specific Genomic Analysis of BDO_2-Induced *hprt* Mutant Human TK6 Cells That Did Not Produce an *hprt*-Specific RT-PCR Product Detectable by Agarose Gel Electrophoresis[a]

Number of BDO_2-Induced Mutations (Total n = 19)	K-*ras*	Exon-Specific Genomic *hprt* PCR Products			
		Exon 1	Exon 2	Exon 4	Exon 9
5	+	—	—	—	—
5	+	—	+	+	+
1	+	—	—	+	+
1	+	—	—	—	+
5	+	+	+	+	—
2	+	+	+	—	—

[a] A — indicates no PCR amplification product; a + indicates PCR amplification product.

Table 5. Internal Deletions and Insertions in BDO_2-Induced *hprt* Mutant Human TK6 Cells

Mutant	Exon Affected	Comments
3D92B	2	Genomic deletion of exon 2; exon 2 absent in cDNA
3D11B	2, 3	Genomic deletion of exon 3; exons 2 and 3 absent in cDNA
3F92A	2, 3	Genomic deletion of exon 3; exons 2 and 3 absent in cDNA
3C21B	4, 5	Genomic deletion of exons 4 and 5; exons 4 and 5 absent in cDNA
3F11B	4, 5, 6	Genomic deletion of exons 4, 5, and 6; exons 4, 5, and 6 absent in cDNA
3E41B	5	Genomic deletion of exon 5; exon 5 absent in cDNA
3F62A	5	Genomic deletion of exon 5; exon 5 absent in cDNA
3D61A	5	Genomic deletion of exon 5; exon 5 absent in cDNA
3F32A	6	46-bp deletion in exon 6 (*hprt* 34,941–34,987)[a]; exon 6 absent in cDNA
3E61A	6, 7, 8	Genomic deletion of exons 6, 7, and 8; exons 6, 7, and 8 absent in cDNA
3B91B	7, 8	Genomic deletion of exons 7 and 8; exons 7 and 8 absent in cDNA
3C81B	7, 8	Genomic deletion of exons 7 and 8; exons 7 and 8 absent in cDNA
3A31A	8	–T at IN7: (40,116); exon 8 absent in cDNA
3C91B	4	Insertion of ATTG after *hprt* cDNA bp 352: TA ATTG352 GT→TA ATTG ATTGGT

[a] The numbering system for the genomic *hprt* gene (Edwards et al. 1990) is given in parentheses.

splicing at presumed cryptic splice sites). Among BDO_2-induced mutants, there were a total of 11 mutations at A•T base pairs and 7 mutations at G•C base pairs; there were 4 transitions and 14 transversions (Tables 3 and 6).

Statistical analysis (one-tailed Fisher exact test) was done on the frequency of each mutational type determined in BDO_2-induced mutants compared to background mutants (Steen et al. 1997a) to assess differences in the mutational spectrum (Table 3). There was a significant ($p < 0.05$) increase in A•T→T•A transversions in BDO_2-induced mutants compared to the background mutants: 9 of 51 (18%) versus 2 of 43 (5%). The frequency of BDO_2-induced mutants with partial 5′ deletions (7 of 51; 14%) was significantly greater ($p = 0.05$) than the frequency among background mutants (1 of 43; 2%).

SPECIFIC AIM 2. THE IN VITRO MUTAGENICITY AND MUTATIONAL SPECTRA OF BDO AND BDO_2 AT THE *lacI* TRANSGENE IN Rat2 CELLS

Cytotoxicity of BDO in Rat2 Cells

Relative survival of Rat2 cells following exposure to a range of concentrations of BDO was determined by recording cumulative cell growth over a period of 7 days (168 hours) following exposure to BDO. Survival relative to medium controls was assessed as a percentage of the

Table 6. A Summary of Base-Substitution Mutations in *hprt* cDNA or in Genomic DNA from the DNA Sequence Analysis of BDO_2-Induced[a] *hprt* Mutant Human TK6 Cells

BDO_2-Induced Mutant	Base Pair Number[b]	DNA Sequence Context[c] 5′ → 3′	Mutant Codon	Base Change	Predicted Amino Acid Change	Exon or Intron Affected	Comments
3C41B	119	CAT GGA CTA	GCA	G→C	Gly→Ala	2	
3A21A	135	GAC AGG ACT	AGT	G→T	Arg→Ser	3	
3A81B	166	AAG GAG ATG	TAG	G→T	Glu→term	3	
3F21A	209	AAG GGG GGC	GAG	G→A	Gly→Glu	3	
3A51B	551	ATT CCA GAC	CTA	C→T	Frameshift[d]	8	Exon 8 skipped in cDNA; mutation identified in exon 8 genomic DNA bp 40,051
3B61B	574	TAT GCC CTT	CCC	G→C	Ala→Pro	8	
3E11B	601	AGG GAT TTG	AAT	G→A	Asp→Asn	8	
3B72A	437	ACT TTG CTT	TAG	T→A	Leu→term	6	
3F82A	449	TTG GTC AGG	GAC	T→A	Val→Asp	6	
3F101A	487	AGC TTG CTG	ATG	T→A	Leu→Met	7	
3E102A	598	TTC AGG GAT	GGG	A→G	Arg→Gly	8	
3B42A	IN1: (1,705)	GTG] gtg agc	gag	T→ A	Frameshift	1	48 bases of intron 1 included in cDNA sequence
3B31A	IN2: (14,888)	CAG] gta agt	gaa	T→A	Frameshift	2	Exon 2 skipped in cDNA
3B81B	IN2: (14,888)	CAG] gta agt	gga	T→G	Frameshift	2	Exon 2 skipped in cDNA
3D42A	IN4: (27,959)	AAG] gta tgt	gaa	T→A	In-frame deletion	4	Exon 4 skipped in cDNA
3A11A	IN5: (34,936)	tga aag [GAT	atg	A→T	Frameshift	5	Exon 6 skipped in cDNA
3F51B	IN6: (35,022)	AAG] gta tgt	gaa	T→A	Frameshift	6	Exon 6 skipped in cDNA
3C71B	IN6: (39,814)	taa cag [CTT	ctg	A→T	Frameshift	6	Exon 7 skipped in cDNA

[a] TK6 cells were exposed to 4 μM BDO_2 for 24 hours.

[b] For *hprt* cDNA, base number 1 is the A in the AUG start codon. For mutations present in intron sequence, the intron affected and the base number are according to Edwards and associates (1990).

[c] Uppercase letters are exon sequence, and lowercase letters are intron sequence.

[d] In mammalian cells, a base-substitution mutation in an intron sequence can result in aberrant splicing of mRNA (exon skipping) that will result in a frameshift in the amino acid codons for *hprt*.

number of cells surviving at 120 hours after plating. Exposure of Rat2 cells to 0.4, 0.6, 0.8, 1.0, or 1.2 mM BDO resulted in 49%, 31%, 11%, 10%, and 5% survival, respectively, compared with medium controls (Figure 2).

Mutagenicity of BDO in Rat2 Cells

Two independent experiments were conducted to determine the mutagenicity of BDO at the *lacI* transgene in Rat2 cells. The results are displayed in Table 7. In the first experiment, cells exposed to medium only or medium containing 0.1% DMSO (solvent control) had mean (± SD) mutant frequencies of 6.6 (± 0.1) × 10^{-5} and 6.5 (± 1.2) × 10^{-5}, respectively. Cells exposed to 0.6 or 1.0 mM BDO had mean (± SD) mutant frequencies of 13.2 (± 3.2) × 10^{-5} and 18.6 (± 1.8) × 10^{-5}, respectively. These values represent increases over background of approximately two- and threefold, which are statistically significant (ANOVA; $p = 0.006$).

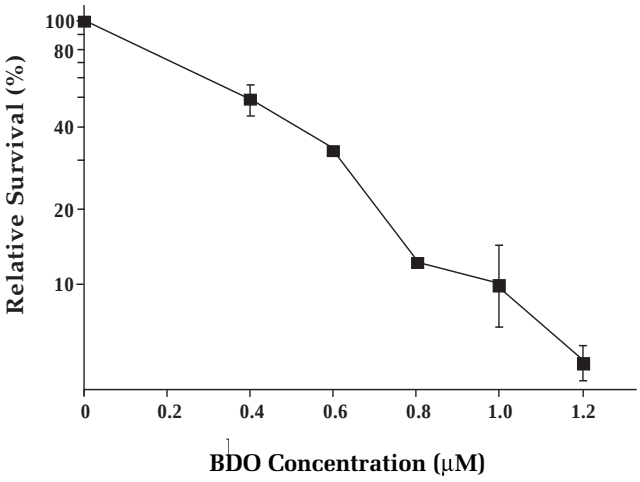

Figure 2. Survival of Rat2 cells (plotted on a logrithmic scale) following 24-hour exposures to specified concentrations of BDO compared with control cells. Data are means ± SD.

In the second experiment, three replicates per exposure concentration were used. Media and solvent controls had mean (± SD) mutant frequencies similar to those of the controls in the first experiment (5.6 [± 0.7] × 10^{-5} and 6.4 [± 0.7] × 10^{-5}, respectively). A dose response (mean ± SD) similar to the first experiment was seen in the cells exposed to 0.6 or 1.0 mM BDO (14.8 [± 0.8] × 10^{-5} and 18.1 [± 5.4] × 10^{-5}, respectively) (ANOVA; $p = 0.001$). In the two experiments, 10 mM ENU, the positive control, induced a mutagenic response.

Table 7. *lacI* Mutant Frequency in Big Blue™ Rat2 Cells Exposed to BDO

Exposure Concentration[a]	*lacI* Mutant Plaques / Total Plaques Screened	*lacI* Mutant Frequency (× 10^{-5})	Treatment Mean (± SD)
Experiment 1			
DMEM	15 / 222,423	6.74	
	14 / 213,706	6.55	6.6 ± 0.1
DMSO	15 / 243,716	6.15	
	18 / 263,679	6.83	6.5 ± 1.2
ENU (10 mM)	235 / 207,107	113.47	
	219 / 198,794	110.16	111.8 ± 2.3
BDO (0.6 mM)	27 / 245,764	10.99	
	38 / 245,270	15.49	13.2 ± 3.2[b]
BDO (1.0 mM)	42 / 214,455	19.58	
	43 / 244,417	17.59	18.6 ± 1.8
Experiment 2			
DMEM	13 / 270,299	4.81	
	17 / 293,725	5.79	
	14 / 227,694	6.15	5.6 ± 0.7
DMSO	18 / 252,070	7.14	
	17 / 272,405	6.24	
	16 / 273,253	5.84	6.4 ± 0.7
ENU (10 mM)	599 / 179,861	333.03	
	553 / 195,834	282.38	
	575 / 205,441	279.89	298.4 ± 30.0
BDO (0.6 mM)	35 / 227,785	15.37	
	43 / 286,086	15.03	
	32 / 229,933	13.92	14.8 ± 0.8[b]
BDO (1.0 mM)	35 / 222,699	15.72	
	39 / 273,801	14.24	
	70 / 288,012	24.30	18.1 ± 5.4[b]

[a] DMEM was the medium control, DMSO was the solvent control, and ENU was the positive control.

[b] Significantly different from medium and solvent controls.

DNA Sequence Analysis of *lacI* Mutants

For determination of the mutational spectra in unexposed Rat2 cells and in Rat2 cells exposed to BDO, mutants from medium and solvent controls from both experiments were selected and collectively considered background, whereas mutants from cells exposed to 1.0 mM BDO in both experiments were selected as the exposed group. Because the MF for this group was approximately threefold that of controls, approximately two mutants out of three should be BDO-induced.

Background *lacI* Mutational Spectrum in Rat2 Cells

From the unexposed control cells, 87 mutants were analyzed by DNA sequencing. For 2 of the 87, no mutation was found within the amplified PCR product. Of the remaining 85 mutants, 73 (86%) were considered to have independent mutations. The types and locations of base substitution mutations are shown in Table 8, and the indicated bases refer to the coding strand of the *lacI* gene. Complex mutations, insertions, and deletions are shown in Table 9. The overall background mutational spectrum, including category-specific mutation frequencies, is summarized in Table 10.

Single base-pair substitution mutations accounted for 79% of all background mutations. In 52 of the 73 mutants (71%), the background mutations occurred at G•C base pairs, with 6 of 73 (8%) occurring at A•T base pairs. G•C→A•T transitions accounted for 24 of 73 (33%) of the background mutations, with 20 of the 24 occurring at 5′-CpG-3′ sites. This category of mutation (G•C→A•T) typically makes a larger contribution to the background mutational spectrum at *lacI*, but in this case, a single mutation (588G→T) was recovered in 21 sequenced mutants. Although 11 of these were considered siblings and excluded from the analysis, this mutation was counted once for each independently exposed sample in which it occurred (10), making the contribution of G•C→T•A transversions unusually high, at 20 of 73 (27%). G•C→C•G transversions accounted for 8 of 73 (11%) of the background *lacI* mutations. Of the 73 mutations at A•T base pairs, 3 (4%) were A•T→G•C transitions, 1 (1%) was an A•T→C•G transversion, and 2 (3%) were A•T→T•A transversions.

From the 73 independent background mutants analyzed, 15 (20%) deletions, insertions, or complex mutations were recovered (Table 9). Most of these were insertions or deletions of a single base that resulted in frameshift mutations.

lacI Mutational Spectrum in Rat2 Cells Exposed to BDO

Of the Rat2 cells exposed to 1.0 mM BDO, 72 mutants were analyzed by DNA sequencing. The types and locations of base-substitution mutations are shown in Table 8 (indicated bases refer to the coding strand of the *lacI* gene).

Table 8. Background and BDO-Induced *lacI* Base-Substitution Mutations in Rat2 Cell *lacI* Mutants

Mutant	Base Pair Number	DNA Sequence Context[a] 5′ → 3′	Mutant Codon	Base Change	Predicted Amino Acid Change	Sibling Mutants[b]
Background Mutations						
EBSS-31	–33	tgg TGC aaa	TAC	G→A		
EBSS-40	42	gta ACG tta	ATG	C→T	Thr→Met	
EBSS-90	56	gtc GCA gag	ACA	G→A	Ala→Thr	
EBSS-97	56	gtc GCA gag	ACA	G→A	Ala→Thr	+
EBSS-103	57	gtc GCA gag	GTA	C→T	Ala→Val	
EBSS-41	80	tat CAG acc	GAG	C→G	Gln→Glu	
EBSS-93	80	tat CAG acc	GAG	C→G	Gln→Glu	
EBMS-20	86	acc GTT tcc	TTT	G→T	Val→Phe	
EBSS-101	89	aaa CAG tcg	TAG	C→T	Gln→term	
EBSS-100	90	gtt TCC cgc	TGC	C→G	Ser→Cys	
EBMS-13	95	gtc TCT tat	TGT	C→G	Ser→Cys	
EBMS-9	99	gtg GTG aac	GAG	T→A	Val→Glu	
EBSS-43	99	gtg GTG aac	GAG	T→A	Val→Glu	
EBMS-59	108	cag GCC agc	GAC	C→A	Ala→Asp	
EBSS-82	120	gtt TCT gcg	TAT	C→A	Ser→Tyr	
EBMS-49	129	aaa ACG cgg	ATG	C→T	Thr→Met	
EBSS-37	129	aaa ACG cgg	ATG	C→T	Thr→Met	
EBSS-25	150	gcg GCG atg	GAG	C→A	Ala→Glu	
EBMS-23	167	aat TAC att	CAC	T→C	Thr→His	
EBMS-51	170	tac ATT ccc	GTT	A→G	Ile→Val	
EBMS-22	180	aac CGC gtg	CAC	G→A	Arg→His	
EBSS-24	180	aac CGC gtg	CAC	G→A	Arg→His	
EBMS-52	185	gtg GCA caa	CCA	G→C	Ala→Pro	
EBMS-79	188	gca CAA caa	GAA	C→G	Gln→Glu	
EBSS-33	197	ctg GCG ggc	TCG	G→T	Ala→Ser	
EBMS-19	198	ctg GCG ggc	GAG	C→A	Ala→Glu	
EBSS-29	201	gcg GGC aaa	GAG	G→A	Gly→Asp	
EBMS-56	269	tgt GCG gcg	ACG	G→A	Ala→Thr	
EBMS-62	270	gtg GCG gcg	GTG	C→T	Ala→Val	
EBMS-69	270	gtg GCG gcg	GTG	C→T	Ala→Val	
EBMS-64	308	acg GGA gtg	ATG	G→A	Val→Met	
EBMS-81	308	acg GGA gtg	ATG	G→A	Val→Met	
EBMS-85	381	caa CGC gtc	CAC	G→A	Arg→His	
EBMS-50	518	cat GAA gac	TAA	G→T	Glu→term	
EBMS-3	530	acg CGA ctg	TGA	C→T	Arg→term	

(*Table continues next page*)

[a] Uppercase letters are exon sequence, lowercase letters are intron sequence.

[b] Sibling mutants are defined as those occurring more than once in the same independently exposed sample (see Materials and Methods section).

[c] The total mutants minus the number of sibling mutants.

[d] Mutant EB1.0-169 had two mutations, which are listed separately by base number.

Table 8. Background and BDO-Induced *lacI* Base-Substitution Mutations in Rat2 Cell *lacI* Mutants (*Continued*)

Mutant	Base Pair Number	DNA Sequence Context[a] 5′ → 3′	Mutant Codon	Base Change	Predicted Amino Acid Change	Sibling Mutants[b]
EBMS-75	530	acg CGA ctg	TGA	C→T	Arg→term	
EBSS-28	575	atc GCG ctg	CCG	G→C	Ala→Pro	
EBMS-1	588	cgc GGC cca	GTC	G→T	Gly→Val	
EBMS-21	588	cgc GGC cca	GTC	G→T	Gly→Val	
EBMS-48	588	cgc GGC cca	GTC	G→T	Gly→Val	
EBMS-55	588	cgc GGC cca	GTC	G→T	Gly→Val	+
EBMS-57	588	cgc GGC cca	GTC	G→T	Gly→Val	
EBMS-63	588	cgc GGC cca	GTC	G→T	Gly→Val	+
EBMS-65	588	cgc GGC cca	GTC	G→T	Gly→Val	+
EBMS-70	588	cgc GGC cca	GTC	G→T	Gly→Val	
EBMS-71	588	cgc GGC cca	GTC	G→T	Gly→Val	+
EBMS-72	588	cgc GGC cca	GTC	G→T	Gly→Val	+
EBSS-27	588	cgc GGC cca	GTC	G→T	Gly→Val	
EBSS-30	588	cgc GGC cca	GTC	G→T	Gly→Val	+
EBSS-35	588	cgc GGC cca	GTC	G→T	Gly→Val	
EBSS-42	588	cgc GGC cca	GTC	G→T	Gly→Val	+
EBSS-44	588	cgc GGC cca	GTC	G→T	Gly→Val	+
EBSS-80	588	cgc GGC cca	GTC	G→T	Gly→Val	
EBSS-84	588	cgc GGC cca	GTC	G→T	Gly→Val	+
EBSS-89	588	cgc GGC cca	GTC	G→T	Gly→Val	
EBSS-91	588	cgc GGC cca	GTC	G→T	Gly→Val	+
EBSS-92	588	cgc GGC cca	GTC	G→T	Gly→Val	+
EBSS-102	588	cgc GGC cca	GTC	G→T	Gly→Val	
EBSS-104	594	cca TTA atg	TGA	T→G	Leu→ term	
EBSS-88	629	ggc TGG cat	CGG	T→C	Trp→Arg	
EBSS-32	777	atg GCG ctg	GTG	C→T	Ala→Val	
EBMS-7	785	ggc GCA atg	CCA	G→C	Ala→Pro	
EBMS-73	791	atg CGC gcc	TGC	C→T	Arg→Cys	
EBMS-12	792	atg CGC gcc	CAC	G→A	Arg→His	
EBMS-83	792	atg CGC gcc	CAC	G→A	Arg→His	
EBMS-61	803	acc GAG tcc	TAG	G→T	Glu→term	
EBMS-8	867	agc TCA tgt	TAA	C→A	Ser→term	
EBMS-15	882	ccg CCG tta	CTG	C→T	Pro→Leu	
EBMS-10	888	tta ACC acc	ATC	C→T	Thr→Ile	
EBSS-95	1206	gag CGG ata	CAG	G→A	Arg→Gln	
Total independent mutants[c]	58					

(*Table continues next page*)

[a] Uppercase letters are exon sequence, lowercase letters are intron sequence.

[b] Sibling mutants are defined as those occurring more than once in the same independently exposed sample (see Materials and Methods section).

[c] The total mutants minus the number of sibling mutants.

[d] Mutant EB1.0-169 had two mutations, which are listed separately by base number.

Table 8. Background and BDO-Induced *lacI* Base-Substitution Mutations in Rat2 Cell *lacI* Mutants (*Continued*)

Mutant	Base Pair Number	DNA Sequence Context[a] 5′ → 3′	Mutant Codon	Base Change	Predicted Amino Acid Change	Sibling Mutants[b]
BDO-Induced						
EB1.0-135	–34	tgg TGC aaa	GGC	T→G		
EB1.0-110	39	cca GTA acg	GAA	T→A	Val→Glu	
EB1.0-165	42	gta ACG tta	AAG	C→A	Thr→Lys	
EB1.0-173	42	gta ACG tta	AAG	C→A	Thr→Lys	+
EB1.0-107	56	gtc GCA gag	CCA	G→C	Ala→Pro	
EB1.0-155	56	gtc GCA gag	CCA	G→C	Ala→Pro	
EB1.0-177	78	tct TAT cag	TTT	A→T	Tyr→Phe	
EB1.0-205	86	acc GTT tcc	CTT	G→C	Val→Leu	
EB1.0-189	89	gtt TCC cgc	GCC	T→G	Ser→Ala	
EB1.0-190	96	cgc GTG gtg	GAG	T→A	Val→Glu	
EB1.0-160	99	gtg GTC aac	GAG	T→A	Val→Glu	
EB1.0-201	99	gtg GTG aac	GAG	T→A	Val→Glu	
EB1.0-200	120	gtt TCT gcg	TTT	C→T	Ser→Phe	
EB1.0-167	129	aaa ACG cgg	AAG	C→A	Thr→Lys	
EB1.0-207	153	gcg ATG gcg	ACG	T→C	Met→Thr	
EB1.0-211	166	ctg AAT tac	AAA	T→A	Asn→Lys	
EB1.0-117	176	ccc AAC cgc	TAC	A→T	Asn→Tyr	
EB1.0-197	178	ccc AAC cgc	AAA	C→A	Asn→Lys	
EB1.0-181	180	aac CGC gtg	CCC	G→C	Arg→Pro	
EB1.0-130	185	gtg GCA cca	ACA	G→A	Ser→Thr	
EB1.0-220	188	gca CAA caa	AAA	C→A	Gln→Lys	
EB1.0-145	194	caa CTG gcg	ATG	C→A	Leu→Met	
EB1.0-159	200	gcg GGC aaa	AGC	G→A	Gly→Ser	
EB1.0-169[d]	237	tcc AGT ctg	ATT	G→T	Ser→Ile	
EB1.0-183	250	ctg CAC gcg	CAA	C→A	His→Gln	
EB1.0-140	260	tcg CAA att	GAA	C→G	Gln→Glu	
EB1.0-185	282	aaa TCT cgc	TAT	C→A	Ser→Tyr	
EB1.0-180	293	tcg CAA att	TAA	C→T	Gln→term	
EB1.0-175	329	gaa CGA agc	GGA	C→G	Arg→Gly	
EB1.0-133	341	gtc GAA gcc	TAA	G→T	Glu→term	
EB1.0-163	369	aat CTT ctc	CGT	T→G	Leu→Arg	
EB1.0-119	372	ctt CTC gcg	CCC	T→C	Leu→Pro	
EB1.0-223	450	tgc ACT aat	ATT	C→T	Thr→Ile	
EB1.0-221	510	att TTC tcc	TCC	T→C	Phe→Ser	
EB1.0-215	530	acg CGA ctg	TGA	C→T	Arg→term	

(*Table continues next page*)

[a] Uppercase letters are exon sequence, lowercase letters are intron sequence.

[b] Sibling mutants are defined as those occurring more than once in the same independently exposed sample (see Materials and Methods section).

[c] The total mutants minus the number of sibling mutants.

[d] Mutant EB1.0-169 had two mutations, which are listed separately by base number.

Table 8. Background and BDO-Induced *lacI* Base-Substitution Mutations in Rat2 Cell *lacI* Mutants (*Continued*)

Mutant	Base Pair Number	DNA Sequence Context[a] 5′ → 3′	Mutant Codon	Base Change	Predicted Amino Acid Change	Sibling Mutants[b]
EB1.0-143	557	gca TTG ggt	ATG	T→A	Leu→Met	
EB1.0-141	582	ctg TTA gcg	TGA	T→G	Leu→term	
EB1.0-109	588	gcg GGC cca	GTC	G→T	Gly→Val	
EB1.0-127	588	gcg GGC cca	GTC	G→T	Gly→Val	+
EB1.0-147	588	gcg GGC cca	GTC	G→T	Gly→Val	
EB1.0-199	588	gcg GGC cca	GTC	G→T	Gly→Val	
EB1.0-203	588	gcg GGC cca	GTC	G→T	Gly→Val	+
EB1.0-217	588	gcg GGC cca	GTC	G→T	Gly→Val	
EB1.0-157	620	cgt CTG gct	ATG	C→A	Leu→Met	
EB1.0-191	629	ggc TGG cat	AGG	T→A	Trp→Arg	
EB1.0-161	653	aat CAA att	TAA	C→T	Gln→term	
EB1.0-129	731	aat GAG ggc	TAG	G→T	Glu→term	
EB1.0-213	783	ctg GGC gca	GTC	G→T	Gly→Val	
EB1.0-179	786	ggc GCA atg	GAA	C→A	Ala→Glu	
EB1.0-121	789	gca ATG cgc	AGG	T→G	Met→Arg	
EB1.0-105	791	atg CGC gcc	AGC	C→A	Arg→Ser	
EB1.0-153	792	atg CGC gcc	CAC	G→A	Arg→His	
EB1.0-125	798	gcc ATT acc	AAT	T→A	Ile→Asn	
EB1.0-139	843	gtg GGA tac	GTA	G→T	Gly→Val	
EB1.0-150	847	gga TAC gac	TAG	C→G	Tyr→term	
EB1.0-131	864	gac AGC tca	ATC	G→T	Ser→Ile	
EB1.0-170	867	agc TCA tgt	TGA	C→G	Ser→term	
EB1.0-111	885	ccg TTA acc	TGA	T→G	Leu→ term	
EB1.0-113	891	acc ACC atc	ATC	C→T	Thr→Ile	
EB1.0-149	930	agc GTG gac	GAG	T→A	Val→Glu	
EB1.0-120	956	cag GGC cag	AGC	G→A	Gly→Ser	
EB1.0-123	1004	aaa AGA aaa	GGA	A→G	Arg→Gly	
EB1.0-137	1012	aaa ACC acc	ATC	C→T	Thr→Ile	
EB1.0-169[d]	1080	gca CGA cag	CCA	G→C	Arg→Pro	
Total independent mutants[c]	61					

[a] Uppercase letters are exon sequence, lowercase letters are intron sequence.

[b] Sibling mutants are defined as those occurring more than once in the same independently exposed sample (see Materials and Methods section).

[c] The total mutants minus the number of sibling mutants.

[d] Mutant EB1.0-169 had two mutations, which are listed separately by base number.

Complex mutations, insertions, and deletions are shown in Table 9, and the overall mutational spectrum from the Rat2 cells exposed to 1.0 mM BDO, including category-specific MFs, is summarized in Table 10.

As with the background mutants, 2 of the 72 BDO-induced mutants contained no mutation within the amplified PCR product. From the 70 mutants, 68 independent mutations were found, of which 62 (91%) were base-substitution mutations. One mutant contained two mutations within the *lacI* gene. Of these 62 mutations, 41 (60%) occurred at G•C base pairs and 2 (31%) occurred at A•T base pairs.

G•C→A•T transitions accounted for 11 of 68 (16%) of the BDO-induced mutants. In contrast to the background mutants, in which most of these mutations occurred at 5′-CpG-3′ sites, most G•C→A•T transitions in BDO-induced mutants (9 of 11) occurred at non 5′-CpG-3′ sites. The frequency of these mutations in the BDO-exposed cells was significantly

increased ($p = 0.003$) when compared to controls (Table 10). G•C→T•A transversions accounted for 22 of 68 (32%) of the BDO-induced mutants, a significant increase ($p < 0.0001$) in the frequency of such mutations over background mutants. There was a significant increase ($p = 0.005$) in the frequency of A•T→C•G transversions among the BDO-induced mutants (6 of 68; 9%) compared with background mutants (1 of 71; 1%). In addition, the frequency of A•T→T•A transversions was significantly increased ($p < 0.0001$) among the BDO-induced mutants (11 of 68; 16%) compared with background mutants (2 of 71; 3%).

Analysis of the 68 mutations from BDO-exposed cells revealed 6 (9%) insertions, deletions, or complex mutations compared with 13 of 71 (18%) such events among background mutants (Table 10).

Cytotoxicity of BDO_2 in Rat2 Cells

Relative survival of Rat2 cells following exposure to 0, 2, 5, or 10 μM BDO_2 was determined by cumulative cell growth over a period of 7 days (168 hours). Survival relative to media controls was expressed as a percentage of the number of cells counted at 120 hours after exposure. Exposure of the cells to 2, 5, and 10 μM BDO_2 resulted in a relative survival (± SD) based on cumulative cell growth of 100% ± 17%, 47% ± 8%, and 13% ± 1%, respectively (Figure 3).

Mutagenicity of BDO_2 in Rat2 Cells

The conditions used to assess mutagenicity of BDO_2 were the same as those described earlier for BDO. Three independent experiments were conducted to determine if BDO_2 was mutagenic at the *lacI* transgene in Big Blue™ Rat2 cells. The results of the three experiments are displayed in Table 11. In the first experiment, cells exposed to medium alone had a (mean ± SD) background MF of 3.9 (± 0.6) × 10^{-5}. Cells

Table 9. Background and BDO-Induced *lacI* Insertions, Deletions, and Complex Mutations in Rat2 Cell Mutants

Type of Mutant	Mutant	Base Pair Number	Mutation	DNA Sequence Context 5′ → 3′	Result
Background Mutations					
Insertions	EBMS-16	70	Ins G	GCC GG$_{\wedge}$T GTG	Frameshift
	EBMS-5	595	Ins T	CCA TT$_{\wedge}$A AGT	Frameshift
	EBSS-39	595	Ins T	CCA TT$_{\wedge}$A AGT	Frameshift
	EBSS-53	595	Ins T	CCA TT$_{\wedge}$A AGT	Frameshift
	EBMS-96	595	Ins T	CCA TT$_{\wedge}$A AGT	Frameshift
	EBMS-76	595	Ins T	TCG GTA$_{\wedge}$ GTG	Frameshift
Deletions	EBMS-17	79	Del T	TCT TA<u>T</u> CAG	Frameshift
	EBSS-77	139	Del A	GAA AA<u>A</u> GTG	Frameshift
	EBSS-36	187–189	Del ACA	GC<u>A CA</u>A CAA	Deletion of Gln
	EBMS-47	192–194	Del AAC	CAA C<u>AA C</u>TG	Deletion of Gln
	EBSS-99	539	Del G	GGC <u>G</u>TG GAG	Frameshift
	EBMS-68	566	Del C	CAC <u>C</u>AG CAA	Frameshift
	EBMS-60	815	Del C	CTG <u>C</u>GC GTT	Frameshift
	EBMS-67	952–953	Del TC	CTC TC<u>T C</u>AG	Frameshift
Tandem Changes	EBSS-87	90–91	CC→TT	GTT T<u>CC</u> CGC	Ser→Phe
BDO-Induced Mutations					
Insertions	EB1.0-115	606	Ins C	GTA T$_{\wedge}$TG GGT	Frameshift
	EB1.0-210	1011	Ins A	AAA A$_{\wedge}$CC ACC	Frameshift
Deletions	EB1.0-187	204–207	Del AACA	GGC A<u>AA C</u>AG	Frameshift
	EB1.0-193	952–953	Del TC	CTC TC<u>T C</u>AG	Frameshift
Complex Mutations	EB1.0-195	541–542, 544–545	Del GG, Ins CC	GT<u>G GA</u>$_{\wedge\wedge}$G CAT→ GTA CCG CAT ($\wedge\wedge$)	Val Glu→ Val Pro
	EB1.0-171	774–805 811–816	Del 37 bp Del 6 bp		

Table 10. Summary of *lacI* Mutations in Rat2 Transgenic Cells

	Background Mutations			BDO-Induced Mutations		
Type of Mutation	Number of Mutations	% of Total	*lacI* Mutant Frequency ($\times 10^{-5}$)	Number of Mutations	% of Total	*lacI* Mutant Frequency ($\times 10^{-5}$)
Transitions						
G•C→A•T	24	34	1.8	11	16	2.9
at CpG	20	28	1.5	2	3	0.5
at non-CpG	4	6	0.3	9	13	2.4[a]
A•T→G•C	3	4	0.2	4	6	1.1
Transversions						
G•C→T•A	20	28	1.5	22	32	5.8[a]
G•C→C•G	8	11	0.6	8	12	2.1
A•T→C•G	1	1	0.1	6	9	1.6[a]
A•T→T•A	2	3	0.1	11	16	2.9[a]
Insertions	6	7	0.4	2	3	0.5
Deletions	7	10	0.5	2	3	0.5
Complex Mutations	1	1	0.1	2	3	0.5
Total	72			68		

[a] $p < 0.01$ using the methods of Carr and Gorelick (1996).

exposed to 2, 5, or 10 µM BDO_2 had mutant frequencies of 3.9 (± 1.7) × 10^{-5}, 6.3 (± 2.8) × 10^{-5}, and 7.1 (± 3.4) × 10^{-5}, respectively. An ANOVA revealed no significant differences between experimental groups ($p = 0.3$), but a linear regression did show a positive trend ($p = 0.06$) toward increasing MF with increasing concentrations of BDO_2.

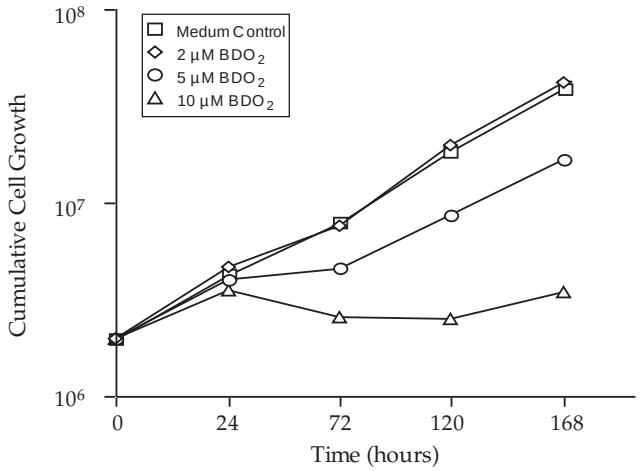

Figure 3. Cumulative growth of Rat2 cells following exposure to specified concentrations of BDO_2 for various durations of time.

In the second experiment, too few cells from the 10 µM BDO_2 treatment group survived to isolate a sufficient quantity of DNA for use in the *lacI* assay. The mean (± SD) background MF was 4.4 (± 0.6) × 10^{-5}, similar to that observed in the first experiment. Cells exposed to 2 or 5 µM BDO_2 had MFs of 5.9 (± 0.4) × 10^{-5}, and 5.8 (± 1.8) × 10^{-5}, respectively. An ANOVA revealed no significant differences between treatments ($p = 0.3$).

In the third experiment, the mean (± SD) background MF was 6.2 (± 0.1) × 10^{-5}, a 1.4- to 1.5-fold increase over the background MFs observed in the other two experiments. However, only two replicates were analyzed, compared to three in each of the previous experiments. Cells exposed to 2, 5, or 10 µM BDO_2 had MFs of 3.4 (± 1.4) × 10^{-5}, 3.7 (± 0.6) × 10^{-5}, and 8.1 (± 2.7) × 10^{-5}, respectively. An ANOVA revealed a significant difference between experimental groups ($p = 0.03$), but a Dunnett multiple-comparison analysis indicated no significant difference (at $\alpha = 0.05$) between these groups and the medium control group. As in the first experiment, a linear regression analysis showed a positive trend ($p = 0.06$) toward increasing MF with increasing BDO_2 concentrations. The trend was weakly positive, however, and the dose-response curve was U-shaped, with the medium controls having higher mutant frequencies than groups exposed to 2 or 5 µM BDO_2.

Table 11. *lacI* Mutant Frequency in Rat2 Cells Exposed to BDO_2

Exposure Concentration[a]	*lacI* Mutant Plaques / Total Plaques Screened	*lacI* Mutant Frequency ($\times 10^{-5}$)	Treatment Mean (± SD)
Experiment 1			
DMEM	9 / 238,834	3.8	
	12 / 266,401	4.5	
	8 / 233,533	3.6	3.9 ± 0.6
DMSO	11 / 236,300	4.7	
	14 / 200,698	6.9	
	13 / 212,033	6.1	5.9 ± 1.2
ENU (10 mM)	174 / 166,034	104.8	
	243 / 207,297	117.2	
	252 / 187,567	134.4	118.8 ± 14.8
BDO_2 (2 μM)	12 / 306,400	3.9	
	5 / 223,236	2.2	
	12 / 217,065	5.5	3.9 ± 1.7
BDO_2 (5 μM)	18 / 198,000	9.1	
	8 / 234,035	3.4	
	13 / 205,402	6.3	6.3 ± 2.8
BDO_2 (10 μM)	22 / 266,791	8.3	
	28 / 286,992	9.8	
	8 / 246,733	3.2	7.1 ± 3.4
Experiment 2			
DMEM	8 / 208,468	3.8	
	11 / 216,667	5.1	
	11 / 249,433	4.4	4.4 ± 0.6
DMSO	7 / 202,300	3.5	3.5
ENU (10 mM)	260 / 233,436	116.4	
	159 / 188,035	84.6	
	353 / 225,408	156.6	117.5 ± 36.4
BDO_2 (2 μM)	11 / 196,399	5.6	
	14 / 218,569	6.4	
	12 / 203,999	5.9	5.9 ± 0.4
BDO_2 (5 μM)	13 / 205,667	6.3	
	10 / 269,039	3.7	
	18 / 247,900	7.3	5.8 ± 1.8
BDO_2 (10 μM)[b]	0 / 0	—	—

(*Table continues next column*)

[a] DMEM was the medium control, DMSO was the solvent control, and ENU was the positive control.

[b] Exposure of Rat2 cells to 10 μM BDO_2 in Experiment 2 resulted in severe cytotoxicity. Insufficient DNA was recovered for use in the *lacI* mutagenesis assay.

Table 11. *lacI* Mutant Frequency in Rat2 Cells Exposed to BDO_2 (*Continued*)

Exposure Concentration[a]	*lacI* Mutant Plaques / Total Plaques Screened	*lacI* Mutant Frequency ($\times 10^{-5}$)	Treatment Mean (± SD)
Experiment 3			
DMEM	13 / 209,432	6.2	
	13 / 213,035	6.1	6.2 ± 0.1
DMSO	15 / 276,400	5.4	5.4
ENU (10 mM)	206 / 203,568	101.2	
	174 / 126,946	137.1	119.1 ± 25.4
BDO_2 (2 μM)	7 / 208,671	3.4	
	10 / 207,429	4.8	
	4 / 203,904	1.9	3.4 ± 1.4
BDO_2 (5 μM)	7 / 231,314	3.0	
	8 / 206,374	3.9	
	7 / 162,776	4.3	3.7 ± 0.6
BDO_2 (10 μM)	22 / 238,984	9.2	
	23 / 236,725	9.7	
	20 / 214,216	9.3	
	10 / 248,054	4.0	8.1 ± 2.7

[a] DMEM was the medium control, DMSO was the solvent control, and ENU was the positive control.

[b] Exposure of Rat2 cells to 10 μM BDO_2 in Experiment 2 resulted in severe cytotoxicity. Insufficient DNA was recovered for use in the *lacI* mutagenesis assay.

Therefore, the positive trend in mutagenicity of BDO_2 in Rat2 cells was driven primarily by the MF at the 10 μM exposure concentration.

Cytokinesis-Block Micronucleus Assay in Rat2 Cells

We examined the induction of micronuclei under similar exposure conditions to the mutation analysis to assess cytogenetic damage due to BDO_2. The Rat2 cells had a background micronuclei frequency (mean ± SD) of 9.9% ± 1.5%. The data in Figure 4 indicate that exposures to 2, 5, and 10 μM BDO_2 for 24 hours all significantly (ANOVA; $p = 0.001$) increased the frequency of micronuclei compared to controls. The mean (± SD) frequencies for these exposure concentrations were 19.3% ± 6.0%, 26.8% ± 6.4%, and 34.4% ± 7.4%, respectively. In the cultures exposed to 0.5 μM BDO_2, micronuclei were not significantly increased. A nuclear division index calculated for all the exposure concentrations (see the section Scoring of Micronuclei in Rat2 Cells under Methods and Study Design) found that only the 10 μM BDO_2 concentration was significantly cytotoxic during the 24-hour exposure period.

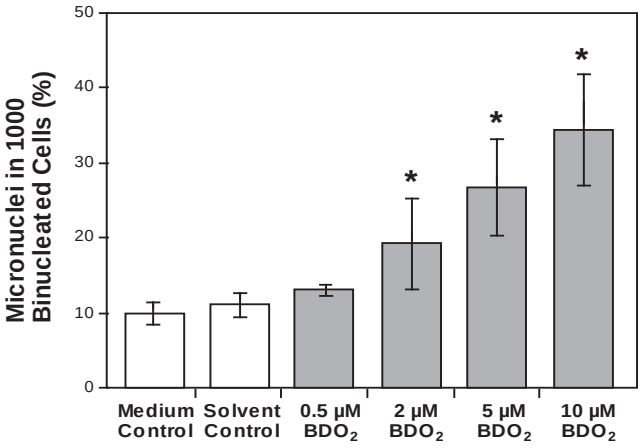

Figure 4. Percentage of micronuclei per 1,000 binucleated Rat2 cells. Cells were counted following 24-hour exposures to medium (DMEM), 0.1% DMSO, or 0.5, 2, 5, or 10 µM BDO_2 for various durations of time. An * indicates a value significantly different ($p < 0.001$) from solvent and medium controls. Data bars are means ± SD.

SPECIFIC AIM 3. THE IN VIVO MUTAGENICITY OF BDO AND BDO_2 IN B6C3F_1 *lacI* TRANSGENIC MICE AND F344 *lacI* TRANSGENIC RATS

Mutant Frequency in Bone Marrow Cells of Transgenic Mice and Rats Exposed to BDO

Exposure of mice to BDO did not increase MF in the bone marrow cells compared with controls exposed only to air. The mean (± SD) *lacI* MF in BDO-exposed mice was 7.8 (± 4.7) × 10^{-5} compared with 8.1 (± 4.1) × 10^{-5} in air-exposed controls (Table 12).

Exposure of rats to BDO increased MF in the bone marrow cells compared with control rats. The mean (± SD) *lacI* MF in BDO-exposed rats was 9.5 (± 1.9) × 10^{-5} compared to 6.4 (± 2.0) × 10^{-5} in controls (Table 13). Although the increase was only 1.5-fold, it was significant (Student *t* test on log-transformed *lacI* MF data according to Callahan and Short [1995]; $p < 0.05$).

Mutant Frequency in Spleen Cells of Transgenic Mice and Rats Exposed to BDO

Exposure of mice to BDO did not increase MF in spleen cells compared with controls. The mean (± SD) *lacI* MF in BDO-exposed mice was 10.7 (± 2.0) × 10^{-5} compared with 9.9 (± 2.8) × 10^{-5} in controls (Table 14).

Exposure of rats to BDO did not increase MF in spleen cells compared with controls. The mean (± SD) *lacI* MF in BDO-exposed rats was 7.7 (± 1.8) × 10^{-5} compared to 6.7 (± 1.4) × 10^{-5} in controls (Table 15).

Table 12. Mutant Frequency in Bone Marrow Cells of *lacI* Transgenic Female Mice Following Inhalation Exposure to BDO

Animal Number	*lacI*$^-$ Plaques / Total Plaques Screened	*lacI*$^-$ Mutant Frequency (× 10^{-5})
0 ppm BDO		
452	14 / 259,447	5.4
454	19 / 225,490	8.4
455	32 / 204,042	15.7
456	11 / 192,156	5.7
457	19 / 213,781	8.9
458	10 / 220,470	4.5
		8.1 ± 4.1[a]
29.9 ppm BDO		
752	26 / 225,700	11.5
754	14 / 256,183	5.5
755	30 / 200,420	15.0
756	19 / 247,627	7.7
757	11 / 197,928	5.6
758	4 / 195,930	2.0
		7.8 ± 4.7[a]

[a] Mean ± SD.

Table 13. Mutant Frequency in Bone Marrow Cells of *lacI* Transgenic Female Rats Following Inhalation Exposure to BDO

Animal Number	*lacI*$^-$ Plaques / Total Plaques Screened	*lacI*$^-$ Mutant Frequency (× 10^{-5})
0 ppm BDO		
209	17 / 204,778	8.3
210	15 / 220,585	6.8
211	7 / 137,041	5.1
212	15 / 228,754	6.6
213	7 / 226,673	3.1
214	17 / 203,264	8.4
		6.4 ± 2.0[a]
29.9 ppm BDO		
241	15 / 228,815	6.6
242	24 / 240,070	10.0
243	24 / 221,788	10.8
244	14 / 166,338	8.4
245	22 / 232,300	9.5
246	25 / 209,840	11.9
		9.5 ± 1.9[a,b]

[a] Mean ± SD.

[b] Significantly different ($p < 0.05$) from controls by Student *t* test using the methods of Callahan and Short (1995).

Table 14. Mutant Frequency in Spleen Cells of *lacI* Transgenic Female Mice Following Inhalation Exposure to BDO

Animal Number	*lacI*$^-$ Plaques / Total Plaques Screened	*lacI*$^-$ Mutant Frequency ($\times 10^{-5}$)
0 ppm BDO		
452	15 / 200,789	7.5
454	20 / 200,895	10.0
455	36 / 237,816	15.1
456	22 / 211,533	10.4
457	18 / 203,163	8.9
458	20 / 271,820	7.4
		9.9 ± 2.8[a]
29.9 ppm BDO		
752	24 / 202,224	11.9
754	23 / 207,809	11.1
755	24 / 196,465	12.2
756	18 / 234,635	7.7
757	27 / 211,884	12.7
758	20 / 229,179	8.7
		10.7 ± 2.0[a]

[a] Mean ± SD.

Table 15. Mutant Frequency in Spleen Cells of *lacI* Transgenic Female Rats Following Inhalation Exposure to BDO

Animal Number	*lacI*$^-$ Plaques / Total Plaques Screened	*lacI*$^-$ Mutant Frequency ($\times 10^{-5}$)
0 ppm BDO		
209	14 / 204,195	6.9
210	13 / 241,458	5.4
211	18 / 201,143	8.9
212	16 / 219,002	7.3
213	12 / 238,858	5.0
214	20 / 291,493	6.9
		6.7 ± 1.4[a]
29.9 ppm BDO		
241	16 / 222,160	7.2
242	21 / 216,484	9.7
243	12 / 202,659	5.9
244	25 / 250,030	10.0
245	17 / 279,317	6.1
246	15 / 215,416	7.0
		7.7 ± 1.8[a]

[a] Mean ± SD.

Mutant Frequency in Lung Cells of Transgenic Mice Exposed to BDO

Exposure of mice to BDO increased more than twofold the *lacI* MF in lung cells compared with control mice (Table 16). The mean (± SD) *lacI* MF in the lungs of BDO-exposed mice was 9.9 (± 3.0) × 10^{-5} compared to 3.6 (± 0.7) × 10^{-5} in controls ($p < 0.01$; Student t test on log-transformed *lacI* MF data according to Callahan and Short [1995]).

Mutant Frequency in Bone Marrow Cells of Transgenic Mice and Rats Exposed to BDO_2

Exposure of mice to BDO_2 did not increase *lacI* MF in bone marrow compared with controls. The mean (± SD) *lacI* MF in BDO_2-exposed mice was 8.1 (± 3.1) × 10^{-5} compared to 5.8 (± 2.6) × 10^{-5} in controls (Table 17).

Exposure of rats to BDO_2 increased MF in bone marrow cells compared with controls. The mean (± SD) *lacI* MF in BDO_2-exposed rats was 6.5 (± 4.2) × 10^{-5} compared to 3.4 (± 0.9) × 10^{-5} in controls (Table 18). Although the increase was less than twofold, it was significant (Student t test on log-transformed *lacI* MF data according to Callahan and Short [1995]; $p < 0.05$).

Table 16. Mutant Frequency in Lung Cells of *lacI* Transgenic Female Mice Following Inhalation Exposure to BDO

Animal Number	*lacI*$^-$ Plaques / Total Plaques Screened	*lacI*$^-$ Mutant Frequency ($\times 10^{-5}$)
0 ppm BDO		
451	11 / 286,261	3.8
452	12 / 337,165	3.6
453	11 / 295,286	3.7
456	10 / 408,283	2.4
457	11 / 323,192	3.4
458	13 / 265,704	4.9
		3.6 ± 0.7[a]
29.9 ppm BDO		
752	13 / 178,583	17.3
754	23 / 207,809	11.1
755	19 / 213,053	8.9
756	31 / 219,201	14.1
757	13 / 211,541	6.1
758	27 / 230,585	11.7
		9.9 ± 3.0[a,b]

[a] Mean ± SD.

[b] Significantly different ($p < 0.05$) from controls by Student t test.

Table 17. Mutant Frequency in Bone Marrow Cells of *lacI* Transgenic Female Mice Following Inhalation Exposure to BDO_2

Animal Number	*lacI*⁻ Plaques / Total Plaques Screened	*lacI*⁻ Mutant Frequency ($\times 10^{-5}$)
0 ppm BDO_2		
744	4 / 76,983	5.2
745	4 / 228,540	1.8
746	14 / 221,461	6.3
747	21 / 233,893	9.0
748	16 / 243,634	6.6
		5.8 ± 2.6[a]
3.8 ppm BDO_2		
771	15 / 215,871	6.9
772	9 / 252,686	3.6
773	18 / 261,706	6.9
774	27 / 230,800	11.7
775	29 / 250,501	11.6
776	16 / 211,591	7.6
		8.1 ± 3.1[a]

[a] Mean ± SD.

Table 18. Mutant Frequency in Bone Marrow Cells of *lacI* Transgenic Rats Following Inhalation Exposure to BDO_2

Animal Number and Sex	*lacI*⁻ Plaques / Total Plaques Screened	*lacI*⁻ Mutant Frequency ($\times 10^{-5}$)
0 ppm BDO_2		
701 M	11 / 241,974	4.5
703 M	10 / 207,340	4.8
704 M	8 / 227,882	3.5
705 M	8 / 245,707	3.3
707 F	6 / 235,253	2.6
709 F	7 / 246,106	2.8
710 F	5 / 215,956	2.3
		3.4 ± 0.95[a]
3.8 ppm BDO_2		
729 M	18 / 255,225	7.1
731 M	19 / 260,306	7.3
732 M	9 / 240,042	3.7
733 M	14 / 243,200	5.8
735 F	6 / 259,840	2.3
736 F	9 / 218,560	4.1
737 F	33 / 204,840	16.1
738 F	13 / 227,921	5.7
		6.5 ± 4.2[a,b]

[a] Mean ± SD.

[b] Significantly different ($p < 0.05$) from controls by Student *t* test.

Table 19. Mutant Frequency in Spleen Cells of *lacI* Transgenic Female Mice Following Inhalation Exposure to BDO_2

Animal Number	*lacI*⁻ Plaques / Total Plaques Screened	*lacI*⁻ Mutant Frequency ($\times 10^{-5}$)
0 ppm BDO_2		
744	17 / 206,012	8.3
745	11 / 203,882	5.4
746	17 / 210,104	8.1
747	15 / 212,809	7.0
748	7 / 214,906	3.3
		6.4 ± 2.1[a]
3.8 ppm BDO_2		
771	15 / 234,680	6.4
772	12 / 205,718	5.8
773	21 / 250,066	8.4
774	22 / 212,489	10.4
775	23 / 216,414	10.6
776	9 / 201,956	4.5
		7.7 ± 2.5[a]

[a] Mean ± SD.

Table 20. Mutant Frequency in Spleen Cells of *lacI* Transgenic Rats Following Inhalation Exposure to BDO_2

Animal Number and Sex	*lacI*⁻ Plaques / Total Plaques Screened	*lacI*⁻ Mutant Frequency ($\times 10^{-5}$)
0 ppm BDO_2		
701 M	18 / 203,321	8.9
703 M	12 / 208,297	5.8
704 M	20 / 248,828	8.0
705 M	15 / 201,785	7.4
707 F	21 / 218,284	9.6
708 F	17 / 222,290	7.6
709 F	12 / 205,422	5.8
710 F	17 / 202,471	8.4
		7.7 ± 1.4[a]
3.8 ppm BDO_2		
729 M	19 / 214,989	8.8
731 M	23 / 233,858	9.8
732 M	17 / 159,881	10.6
733 M	12 / 205,699	5.8
735 F	8 / 204,130	3.9
736 F	19 / 193,035	9.8
737 F	28 / 219,770	12.7
738 F	29 / 229,330	12.6
		9.3 ± 3.1[a]

[a] Mean ± SD.

Mutant Frequency in Spleen Cells of Transgenic Mice and Rats Exposed to BDO_2

Exposure of mice to BDO_2 did not increase MF in spleen cells compared with controls. The mean (± SD) *lacI* MF in BDO_2-exposed mice was 7.7 (± 2.5) × 10^{-5} compared with 6.4 (± 2.1) × 10^{-5} in controls (Table 19).

Exposure of rats to BDO_2 did not increase MF in spleen cells compared with controls. The mean (± SD) *lacI* MF in BDO_2-exposed rats was 9.3 (± 3.1) × 10^{-5} compared with 7.7 (± 1.4) × 10^{-5} in controls (Table 20).

DISCUSSION AND CONCLUSIONS

SPECIFIC AIM 1. THE IN VITRO MUTAGENICITY AND MUTATIONAL SPECTRA OF BDO AND BDO_2 AT *hprt* IN HUMAN TK6 CELLS

The studies to achieve this specific aim examined the in vitro mutational spectra of BDO and BDO_2 in human cells. The metabolites were tested directly in human cells, and the mutational spectra were determined at *hprt*. Although previous analysis of BDO- and BDO_2-induced *hprt* mutants in human TK6 cells examined gross alterations using Southern blot analysis (Cochrane and Skopek 1994a), data were lacking on the frequency of these alterations among background mutants. For the present studies, *hprt* mutants representing background *hprt* mutations were analyzed from the same stock cultures of human TK6 cells, enabling direct comparison with the mutations observed in BDO- and BDO_2-exposed cultures (Steen et al. 1997a,b). Although the mutational spectra observed can be affected by treatment parameters, all attempts were made to ensure that cell culture conditions, exposure conditions, and cell survival conditions were as similar as possible (Chen and Thilly 1996).

An attempt was made to classify all *hprt* mutants at the DNA sequence level, but deletion endpoints in *hprt* mutants were not determined. Deletions were assessed by the absence or presence of specific *hprt* exons in the genomic DNA as determined by PCR amplification analysis. Intron-exon junction sites were sequenced in mutants displaying aberrant mRNA splicing (exon loss) to examine donor and acceptor splice site sequences for mutation.

The BDO mutational spectrum (induced by a concentration of 400 µM BDO for 24 hours) at *hprt* in TK6 cells was analyzed and statistically compared with background *hprt* mutants (Steen et al. 1997a) collected from the same stock cultures. Exposure of human cells to BDO increased the frequency of G•C→A•T transitions, A•T→G•C transitions, and A•T→T•A transversions. The most striking result of BDO exposure was a 19-fold increase in A•T→T•A transversions. The DNA sequence context of the G•C→A•T transitions and mutations at A•T base pairs among the BDO-induced mutants showed strand bias for a purine in the noncoding strand. In 12 of 14 (86%) of the G•C→A•T transitions, and in 19 of 21 (90%) of the mutations observed at A•T base pairs, the G or A was located in the nontranscribed strand; all of the A•T→T•A transversions occurred with the A in the nontranscribed strand. This strand specificity has been described for lesions induced by ultraviolet light at *hprt* in Chinese hamster cells and is associated with the formation and slow repair of pyrimidine dimers in the nontranscribed strand (Vrieling et al. 1991). These data suggest a potential role for purine-derived adducts (guanine or adenine) in the induction of mutation in BDO-exposed human cells. These data also suggest that relevant biomarkers for BD exposure in humans should include BD-derived adducts at guanine or adenine.

The mutation spectrum induced in TK6 cells exposed to BDO_2 (4 µM for 24 hours) was statistically compared to the mutation spectrum among background mutants isolated from the same stock cultures. These analyses indicated that there was an increased frequency of deletions affecting the 5′ region of *hprt*. In five of the BDO_2-induced mutants and in none of the background mutants, exon-specific genomic PCR indicated the presence of exon 2 but the absence of exon 1. Since the distance between exon 1 and 2 is approximately 13.3 kilobases, BDO_2 exposure of human cells can apparently induce genomic deletions at *hprt* that are detectable at the molecular level. The induction of genomic deletions at *hprt* is consistent with the induction of chromosomal alterations in BDO_2-exposed mammalian cells. Among the BDO_2-induced mutants, A•T→T•A transversions were more frequent than among background mutants. However, in contrast to the strand bias observed among the BDO-induced mutants, only 27% of the A•T→T•A transversions among the BDO_2-induced mutants had the adenine in the nontranscribed strand. These data indicate that although both BDO and BDO_2 increase the frequency of A•T→T•A transversions, they induce this mutation by distinct mechanisms.

SPECIFIC AIM 2. THE IN VITRO MUTAGENICITY AND MUTATIONAL SPECTRA OF BDO AND BDO_2 AT THE *lacI* TRANSGENE IN Rat2 CELLS

Rat2 cells were exposed to BDO to assess its mutagenicity and the mutational spectrum at the *lacI* transgene. Mutagenicity experiments demonstrated that exposure of Rat2 cells to BDO at 0.6 and 1.0 mM for 24 hours resulted in a two- and threefold increase in MF, respectively, compared to controls. DNA sequence analysis of mutants recovered in the mutagenicity experiments revealed that 79% of the background mutations and 91% of the BDO-induced mutations were base substitutions. DNA sequencing revealed that among the

background mutants, a single mutation (588G→T) occurred at a high frequency. In fact, its contribution to the background *lacI* MF in these cells was 25.3%. Therefore, if the mean background *lacI* MF were corrected for the frequency of this mutation, the background would be lowered from 6.3 to 4.7 ($\times 10^{-5}$). Because the Rat2 cell line is a relatively new experimental system, there are few historical data on the background MF, but published reports indicate that it ranges from 2.8 to 8.5 $\times 10^{-5}$ (Manjanatha et al. 1996; Suri et al. 1996). In the mutants recovered from cells exposed to 1.0 mM BDO, this mutation (588G→T) was found at least once in 4 of 5 samples. However, its contribution to the BDO MF was approximately threefold lower than in the background mutants.

The background *lacI* mutational spectra generated in these experiments is consistent with spectra previously reported for Rat2 cells (Manjanatha et al. 1996; Suri et al. 1996) and for unexposed *lacI* mice (Kohler et al. 1991; Sisk et al. 1994; Gorelick et al. 1995). G•C→A•T transitions at CpG dinucleotides are the predominant mutation in the background spectrum. Transitions at these sites result from the hypermethylation of cytosine residues in the *lacI* transgene by mammalian DNA methylases. Subsequent deamination of the 5-methylcytosine at CpG sites yields thymine, giving rise to G•C→A•T transitions (Sisk et al. 1994; Gorelick et al. 1995). Other base-substitution mutations occurred at frequencies comparable to other published reports (Kohler et al. 1991; Sisk et al. 1994; Gorelick et al. 1995). Interestingly, background deletion mutations appear to occur at higher frequency in the Rat2 cells than in untreated mice.

The spectrum of *lacI* mutations among BDO-induced mutants was significantly different from the background *lacI* spectrum. The G•C→A•T transitions that occurred with low frequency at non-CpG sites in control cells occurred with increased frequency in BDO-exposed cells. A similar increase in the frequency of this mutational type was seen in the bone marrow and spleen cells of *lacI* mice following exposure to 1,250 ppm BD (Recio et al. 1996, 1998). The frequency of G•C→T•A transversions was also increased in BDO-exposed cells compared to controls. A similar increase in these specific mutations was observed in the spleen cells of *lacI* mice exposed to BD (Recio et al. 1998).

Mutations at A•T base pairs in BDO-induced Rat2 cell mutants increased in frequency in comparison with background mutants, as did A•T→C•G transversions. However, the latter mutation has not been found to increase in frequency in mice following exposure to BD (Sisk et al. 1994; Recio et al. 1996; 1998). One of the most significant differences between the background and BDO-induced spectra was the increased frequency of A•T→T•A transversions in cells exposed to BDO. An increased frequency of A•T→T•A transversions was observed in *lacI* mutants recovered from the spleen and bone marrow cells of *lacI* mice exposed to 625 and 1,250 ppm BD (Sisk et al. 1994; Recio et al. 1996, 1998). Since this mutation was also increased at *hprt* in human TK6 lymphoblasts exposed to BDO (Steen et al. 1997b), it is a consistent result of exposure either to BD in vivo or to BDO in vitro.

Results from these experiments in Rat2 *lacI* transgenic fibroblasts indicate a role for BDO in inducing specific point mutations at the *lacI* transgene. Three types of mutations, including G•C→A•T transitions, G•C→T•A transversions, and A•T→T•A transversions, have been observed to increase in Rat2 *lacI* transgenic cells exposed to BDO. Two of these mutational types, G•C→A•T transitions and A•T→T•A transversions, have been observed in human TK6 cells exposed to BDO (Steen et al. 1997b). All three mutational types have been observed to occur at an increased frequency following inhalation exposure of mice to BD.

BDO_2 was not mutagenic at 2 or 5 µM in Rat2 cells, although weak or marginal mutagenicity was observed at the highest concentration (10 µM). The relevance of the increase in Rat2 cells at the 10 µM BDO_2 exposure concentration to the increase in *lacI* MF in transgenic mice exposed to BD is uncertain (Recio et al. 1996, 1997, 1998). An exposure level of 10 µM BDO_2 is approximately four times higher than levels of BDO_2 found in the blood of mice following inhalation exposure to 1,250 ppm BD (Himmelstein et al. 1994).

The results obtained from the BDO_2 mutagenicity experiments lead us to conclude that BDO_2 is not mutagenic at the *lacI* transgene in vitro at exposure concentrations that result in low to moderate cytotoxicity and is, at most, only weakly mutagenic at concentrations that result in severe cytotoxicity (≤ 10% survival relative to unexposed controls). These results were not altogether surprising due to the clastogenic potential of BDO_2 and the mounting evidence that lambda shuttle vector–based transgenic systems are relatively insensitive to clastogenic events. For example, a lambda shuttle vector–based system was relatively inefficient at detecting x-ray–induced mutations compared to background mutations at the *dbl*-1 locus in Big Blue™ mice (Tao et al. 1993). In addition, mitomycin C, a known clastogen, had little effect on MF at the *lacZ* transgene in the liver and bone marrow cells of *lacZ* transgenic mice in vivo. However, the same mice had significantly elevated levels of micronuclei in the peripheral blood, indicating that the lambda shuttle vector–based *lacZ* transgene does not detect the types of mutagenic events that give rise to micronuclei (Suzuki et al. 1993). Its insensitivity is related to several constraints on the in vitro packaging of the lambda shuttle vector,

including packaging size constraints, and to the need for intact *cos* sites for excision of the lambda phage genome from the host DNA (Gossen et al. 1995).

To confirm that genotoxicity could be detected at the BDO_2 exposure levels used to determine MF in this in vitro system, we used the cytokinesis-block micronucleus assay. A cytogenetic test based on the scoring of chromosomes or chromosome fragments that have separated from the parent nuclei and are encapsulated in small micronuclei, this assay has become a widely accepted method to assess cytogenetic damage (reviewed by Heddle et al. 1991). Our results indicate that BDO_2 induced a dose-dependent increase in micronuclei at exposure concentrations of 2 to 10 µM for 24 hours. These data demonstrate that BDO_2 produces cytotoxicity and cytogenetic damage at concentrations that do not increase the MF at the *lacI* transgene in Rat2 cells, probably due to the inability of this lambda shuttle vector–based system to detect large deletions. The Big Blue™ *lacI* transgenic system is thus likely to prove inadequate for the assessment of the contributions of deletions relative to point mutations for the overall mutational spectrum of BD.

SPECIFIC AIM 3. THE IN VIVO MUTAGENICITY OF BDO AND BDO_2 IN $B6C3F_1$ *lacI* TRANSGENIC MICE AND F344 *lacI* TRANSGENIC RATS

Mutagenicity experiments in transgenic mice have shown that exposure to BD by inhalation at levels that induce tumors in $B6C3F_1$ mice induces in vivo mutation in multiple tissues (Recio et al. 1992; Sisk et al. 1994; Recio et al. 1996, 1997). In Mutamouse™ ($CD2F_1$ *lacZ* transgenic mice), exposure to 625 ppm BD (6 hours/day for 5 days) induced a significant increase in the *lacZ* MF in isolated lung DNA (Recio et al. 1992). In $B6C3F_1$ *lacI* transgenic (Big Blue™) mice exposed to 62.5, 625, and 1,250 ppm BD (6 hours/day, 5 days/week for 4 weeks), a significant increase in the *lacI* MF in the bone marrow and in the spleen cells was observed at all exposure levels (Sisk et al. 1994; Recio et al. 1997). A significant increase in the *lacI* MF also occurred in the bone marrow cells of $B6C3F_1$ *lacI* transgenic mice following a 5-day exposure to 625 ppm BD (Recio et al. 1996).

In the present study, *lacI* mice and rats were exposed by inhalation to BDO and BDO_2 at levels chosen to achieve blood levels equivalent to those that occur in mice exposed to 625 ppm (for BDO) and 62.5 ppm (for BDO_2) of the parent compound BD. Animals were exposed to each metabolite for two weeks. The bone marrow in mice and rats was sampled two weeks after the exposure period, as was mouse spleen; rat spleen was sampled five weeks after the exposure period. These fixation times were based on studies in *lacI* mice exposed to BD (Sisk et al. 1994; Recio et al. 1996, 1997) and mutagenicity studies at *hprt* in spleen of rats exposed to BD (Meng et al. 1999).

In *lacI* mice and rats exposed to BDO (29.9 ppm for 2 weeks), mutagenicity was not detectable in cells of mouse bone marrow, mouse spleen, or rat spleen. However, BDO was mutagenic in the lung cells of mice (higher than twofold increase in the *lacI* MF) and in the bone marrow cells of rats (less than twofold increase in the *lacI* MF). Both increases can be attributed to the direct mutagenicity of BDO exposure, because BDO exposure in vitro is mutagenic to *lacI* transgenic rat cells. The lack of detectable mutagenicity from exposure to BDO in the other three tissue samples may be due to a number of variables, including efficient detoxification of BDO in exposed mice and rats, inappropriate route of exposure, and insufficient exposure levels of BDO in the circulating blood. The finding that BDO was mutagenic in mouse lungs (a target organ for tumor induction) but not in mouse spleen or bone marrow points to detoxification of BDO.

In BDO_2-exposed (3.8 ppm for 2 weeks) *lacI* mice and rats, mutagenicity was not detectable in mouse bone marrow, mouse spleen, or rat spleen. The lack of detectable mutagenicity in BDO_2-exposed mice is probably due to efficient detoxification of BDO_2 in mice, or to poor recovery of point mutations by BDO_2 at the *lacI* transgene in this lambda phage–based mutagenicity assay, as demonstrated in Specific Aim 2, or both. Significant mutagenicity (less than twofold) from BDO_2 exposure was detected in the bone marrow of rats. Although BDO_2 was not mutagenic in Rat2 *lacI* transgenic cells, the one-time 24-hour exposure in vitro versus the subchronic 2-week exposure in vivo makes extrapolation or comparison difficult. Analysis of the *lacI* MF in the lungs of BDO_2-exposed mice is needed to interpret the *lacI* MF data in the tissues of BDO_2-exposed rats.

IMPLICATIONS

Since BD bioactivation can produce at least two genotoxic metabolites, the mutagenicity and mutational spectrum of each metabolite must be studied independently to assess the role of each in the in vivo mutagenicity and mutational spectrum of the parent compound BD.

The studies reported here used commercially available racemic mixtures of BDO and BDO_2 to assess the direct mutagenicity of these metabolites in human and rodent cells. The stereochemistry of these BD metabolites and their production in a series-specific or tissue-specific manner is likely a significant factor that will affect their biological activity and their roles in the mutagenicity and carcinogenicity of the parent compound, BD. In studies with mouse and rat liver microsomes, differences between

species have ben noted in the proportions of the (*R*)-BDO and (*S*)-BDO stereoisomers produced metabolically, and in rat hepatocytes the (*R*)-BDO stereoisomer was more cytotoxic than the (*S*)-BDO stereoisomer (Nieusma et al. 1997). In the same study, *meso*-BDO_2 was apparently more cytotoxic than the racemic mixture of BDO_2 (Nieusma et al. 1997). Because the present studies used commercially available racemic mixtures of BDO and BDO_2, the contributions of specific BD stereoisomers to the observed cytotoxicity, genotoxicity, and mutagenicity in human and rodent cells is uncertain.

The analysis of the mutational spectrum of each metabolite compared with that obtained for the parent compound in vivo is an approach toward identifying the relative mutagenicity of the pertinent BD metabolites. Analysis may also provide mechanistic insights into BD-induced genotoxicity and a basis for developing relevant biomarkers for BD-induced genotoxic effects. The present studies, using three experimental systems to assess the mutagenicity and mutational spectra of BDO and BDO_2, demonstrate that each metabolite has its distinct spectrum of mutations. However, consistent experimental observations were made regarding the patterns of genotoxic effects induced by BDO and BDO_2 in the biological systems analyzed.

To achieve Specific Aim 1, we determined the *hprt* mutational spectra for BDO and BDO_2 in human TK6 cells in vitro (Steen et al. 1997a,b). Exposure of TK6 cells to BDO increased the frequency of G•C→A•T transitions and A•T→G•C transitions and, most strikingly, A•T→T•A transversions (19-fold) compared to unexposed TK6 cells. A strand bias for the G•C→A•T transitions and A•T→T•A transversions observed in BDO-exposed human cells suggests a role for purine adducts (guanine and adenine) in mediating these mutations. An increased frequency of A•T→T•A transversions was also observed at *hprt* in BDO_2-exposed human TK6 cells, but strand bias was not observed. Exposure to BDO_2 induced an increased frequency of deletions of *hprt*. These data indicate that at *hprt* in human cells, BDO induces genotoxicity primarily through point mutations (e.g., A•T→T•A transversions), whereas BDO_2 induces both point mutations and partial deletions.

To achieve Specific Aim 2, the mutagenicity and mutational spectra of BDO and BDO_2 were assessed in Rat2 cells in vitro, using the *lacI* transgene as a mutational target. Exposure of Rat2 cells to BDO increased the frequency of three types of mutations: G•C→A•T transitions, G•C→T•A transversions, and A•T→T•A transversions. Two of these, G•C→A•T transitions and A•T→T•A transversions, were observed in TK6 cells exposed to BDO (Steen et al. 1997b). More important, all three mutational types have been observed to occur at increased frequency following exposure of mice to BD by inhalation (Recio et al. 1998). Exposure of Rat2 cells to BDO_2 increased the micronuclei in Rat2 cells at exposure levels that did not induce significant mutagenicity at the *lacI* transgene (Saranko and Recio 1998). The lack of detectable mutagenicity at the *lacI* transgene in Rat2 cells exposed to BDO_2 is probably due to the poor recovery of large deletions using this lambda phage–based mutagenicity assay (Gossen et al. 1995). These data indicate that BDO induces genotoxicity in Rat2 cells primarily through point mutations (including A•T→T•A transversions), whereas BDO_2 induces micronuclei (chromosomal alterations) at exposure levels that do not induce point mutations.

To achieve Specific Aim 3, the mutagenicity in vivo of BDO and BDO_2 was assessed at the *lacI* transgene following inhalation exposures. Significant mutagenicity (more than twofold) was observed in the lungs of exposed mice and in one tissue sample among the four obtained from BDO-exposed *lacI* mice and rats. In rats, weak mutagenicity (less than twofold greater than controls) was observed in the cells of bone marrow but not spleen. The lack of detectable mutagenicity in the other three tissue samples examined in the present studies may be due to efficient detoxification of BDO in exposed mice (Csanády et al. 1992) resulting in insufficient levels of BDO in the circulating blood. The exposure level of BDO used in the present studies was expected to produce blood levels that correspond to a 625 ppm exposure of BD, which induces in vivo mutation in *lacI* mice. The lack of detectable mutagenicity from exposure to BDO in the present studies is thus likely due to result from detoxification of BDO.

Inhalation exposure to BDO_2 did not induce significant mutagenicity in any of the tissues examined in mice. It was not mutagenic in the spleen of rats, in which it induced an increase (less than twofold) in the *lacI* MF. The exposure level of BDO_2 was expected to produce blood levels of BDO in mice that correspond to a 62.5-ppm exposure of BD, a level that is mutagenic in the bone marrow and spleen of BD-exposed mice (Sisk et al. 1994; Recio et al. 1997). The lack of detectable mutagenicity in BDO_2-exposed mice and rats is probably due to poor recovery of point mutations by BDO_2 at the *lacI* transgene in this lambda phage–based mutagenicity assay, as demonstrated in Specific Aim 2, or to detoxification of BDO_2. Analysis of the *lacI* MF in lung cells is needed to make this distinction.

As indicated previously, in *lacI* mice exposed to BD there is an increased frequency of point mutations at G•C and A•T base pairs in the *lacI* transgene, including an increased frequency of A•T→T•A transversions in bone marrow and spleen cells (Sisk et al. 1994; Recio and Meyer 1995; Recio et al. 1997). The data in this and other studies clearly indicate

that BDO and BDO_2 differ in the mechanisms by which they induce mutation in mammalian cells. In the present studies, BDO specifically induced G•C→A•T and A•T→T•A transversions in vitro at both the endogenous *hprt* gene and the *lacI* transgene in Rat2 cells. It also increased the frequency of G•C→T•A transversions in Rat2 cells. The types of mutations induced by BDO in the present studies also occur at an increased frequency in mice exposed to the parent compound BD. This finding demonstrates the induction of consistent mutational types across biological systems by BDO and indicates that BDO, but not BDO_2, probably has a role in mediating the type of mutations that are recovered at the *lacI* transgene in animals exposed to the parent compound BD. This argument is strengthened by the lack of detectable BDO_2 mutagenicity at the *lacI* transgene in vitro in Rat2 cells and in vivo in transgenic mice. The clastogenic effects of BDO_2 have been well documented (Himmelstein et al. 1997) and are demonstrated in the present studies by an increased frequency of *hprt* deletions in human TK6 cells and by the induction of micronuclei in Rat2 cells. Therefore, it is apparent that in mice exposed to BD at carcinogenic levels, both BDO and BDO_2 have a role in mediating the range of genotoxic responses observed (Figure 5). In the experimental systems examined in the present studies, BDO acts primarily by the induction of point mutations, whereas BDO_2 induces deletions and chromosomal alterations as a primary mode of genotoxicity.

Exposure of mice to BD results in a wide range of genotoxic events that include large-scale rearrangements of DNA and the induction of single-base substitutions. The induction of both of these genotoxic events probably contributes to the overall mutagenicity and carcinogenicity of BD exposure. Whether large deletions or rearrangements or single-base substitution mutations predominate at the low levels of BD exposure normally encountered by humans is uncertain. The data in the present study and other published data indicate that human biomonitoring of BD-exposed populations with the *hprt* mutant T lymphocyte cloning assay (Albertini et al. 1982; Ward et al. 1994, 1996; Hayes et al. 1996) should include the molecular analysis of *hprt* deletions as well as the spectrum of specific base-substitution mutations. In addition, relevant biomarkers of BD exposure, particularly DNA adducts, should be able to predict the range of genotoxic events known to be induced by exposure to BD and its metabolites.

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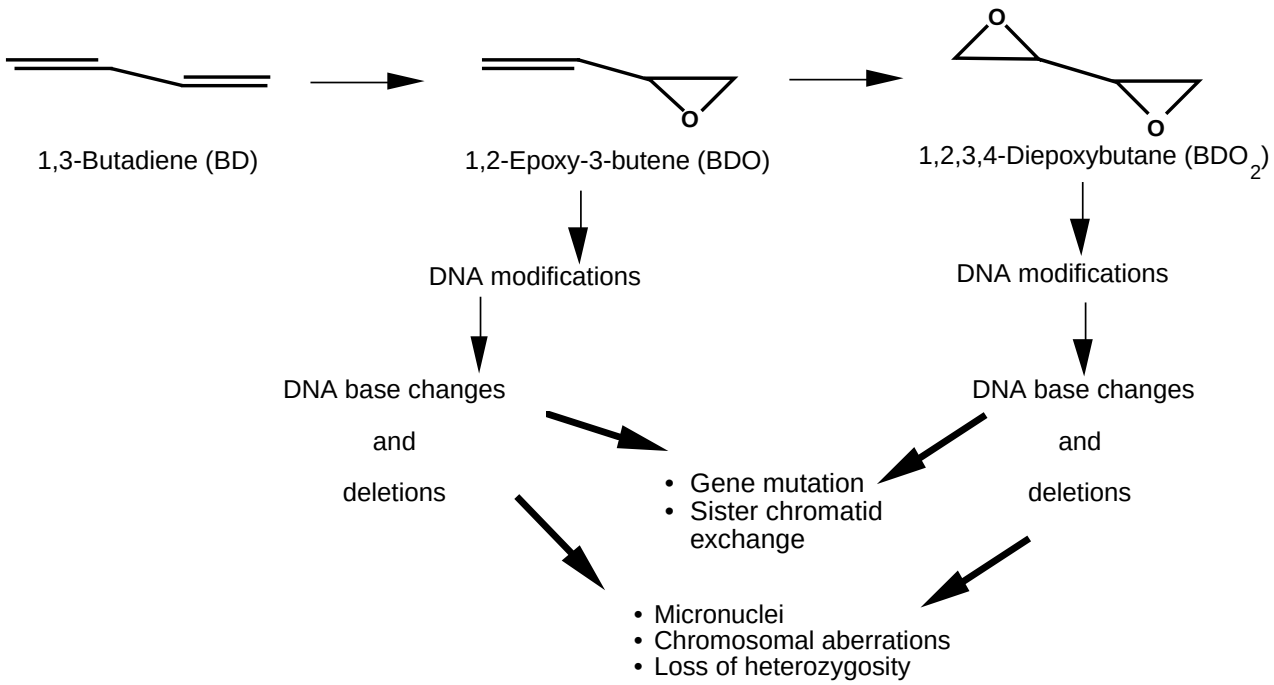

Figure 5. Proposed mechanisms of genotoxicity induced by BDO and BDO_2. (Adapted from Recio et al. 1997 with permission from the Chemical Industry Institute of Toxicology.)

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REFERENCES

Adler ID, Anderson D. 1994. Dominant lethal effects after inhalation exposure to 1,3-butadiene. Mutat Res 309:295–297.

Adler ID, Cao J, Filser JG, Gassner P, Kessler W, Kliesch U, Neuhäuser-Klaus A, Nüsse M. 1994. Mutagenicity of 1,3-butadiene inhalation in somatic and germinal cells of mice. Mutat Res 309:295–297.

Adler ID, Cochrane J, Osterman-Golkar S, Skopek TR, Sorsa M, Vogel E. 1995a. 1,3-Butadiene working group report. Mutat Res 330:101–114.

Adler ID, Filser JG, Gassner P, Kessler W, Schöneich J, Schriever-Schwemmer G. 1995b. Heritable translocations induced by exposure of male mice to 1,3-butadiene. Mutat Res 347:121–127.

Adler ID, Kliesch U, Tiveron C, Pacchierotti F. 1995c. Clastogenicity of 1,2,3,4-diepoxybutane in bone marrow cells and male germ cells of mice. Mutagenesis 10:535–541.

Albertini RJ, Castle KL, Borcherding WR. 1982. T-cell cloning to detect the mutant 6-thioguanine-resistant lymphocytes present in human peripheral blood. Proc Natl Acad Sci USA 79:6617–6621.

Anderson D, Dobrzynka MM, Jackson LI, Yu TW, Brinkworth MH. 1997. Somatic and germ cell effects in rats and mice after treatment with 1,3-butadiene and its metabolites, 1,2-epoxybutene and 1,2,3,4-diepoxybutane. Mutat Res 391: 233–242.

Autio K, Renzi L, Catalan J, Albrecht OE, Sorsa M. 1994. Induction of micronuclei in peripheral blood and bone marrow erythrocytes of rats and mice exposed to 1,3-butadiene by inhalation. Mutat Res 309:315–320.

Bond JA, Recio L, Andjelkovich D. 1995. Epidemiological and mechanistic data suggest that 1,3-butadiene will not be carcinogenic to humans at exposures likely to be encountered in the environment or workplace. Carcinogenesis 16:165–171.

Callahan JD, Short JM. 1995. Transgenic λ /*lacI* mutagenicity assay: Statistical determination of sample size. Mutat Res 327: 201–208.

Carr GJ, Gorelick NJ. 1996. Mutational spectra in transgenic animal research: Data analysis and study design based upon the mutant or mutation frequency. Environ Mol Mutagen 28:405–413.

Chemical and Engineering News. 1994. Top 50 chemical production rose modestly last year. April 11, 2–16.

Chen J, Thilly WG. 1996. Mutational spectra vary with exposure conditions: Benzo[a]pyrene in human cells. Mutat Res 357:209–217.

Citti L, Gervasi PG, Turchi G, Bellucci G, Bianchini R. 1993. The reaction of 3,4-epoxy-1-butene with deoxyguanosine and DNA in vitro: Synthesis and characterization of the main adducts. Carcinogenesis 5(1):47–52.

Cochrane JE, Skopek TR. 1994a. Mutagenicity of butadiene and its epoxide metabolites: I. Mutagenic potential of 1,2-epoxybutene, 1,2,3,4-diepoxybutane and 3,4-epoxy-1,2-butanediol in cultured human lymphoblasts. Carcinogenesis 15:713–717.

Cochrane JE, Skopek TR. 1994b. Mutagenicity of butadiene and its epoxide metabolites: II. Mutational spectra of butadiene, 1,2-epoxybutene and 1,2,3,4-diepoxybutane at the *hprt* locus in splenic T cells from exposed $B6C3F_1$ mice. Carcinogenesis 15:719–723.

Csanády GA, Guengerich FP, Bond JA. 1992. Comparison of the biotransformation of 1,3-butadiene and its metabolite butadiene monoepoxide by hepatic and pulmonary tissues from humans rats and mice. Carcinogenesis 13:1143–1153.

Cunningham A, Sariff AM. 1986. In vivo sister chromatid exchange and micronucleus induction studies with 1,3-butadiene in $B6C3F_1$ mice and Sprague-Dawley rats. Mutagenesis 1:449–452.

Delzell E, Sathiakumar N, Hovinga M, Macaluso M, Julian J, Larson R, Cole P, Muir DCF. 1996. A follow-up study of synthetic rubber workers. Toxicology 113:182–189.

de Meester C. 1988. Genotoxic properties of 1,3-butadiene. Mutat Res 195:273–281.

DeWitt & Co., Houston, TX [Source]. 1996. Markets & economics, product focus: Butadiene. Chem Week August 7, 1996:32.

Duescher RJ, Elfarra AA. 1994. Human liver microsomes are efficient catalysts of 1,3-butadiene oxidation: Evidence for major roles by cytochromes P450 2A6 and 2E1. Arch Biochem Biophys 311:342–349.

Eastmond DA, Tucker JD. 1989. Kinetochore localization in micronucleated cytokinesis-blocked Chinese hamster ovary cells: A new and rapid assay for identifying aneuploidy-inducing agents. Mutat Res 224:517–525.

Edwards A, Voss H, Rice P, Civitello A, Stegman J, Schwager C, Zimmerman J, Erfle H, Caskey C, Ansorge W. 1990. Automated DNA sequencing of the human HPRT locus. Genomics 6:593–608.

Farabaugh PJ, Schmeissner U, Hofer M, Miller JH. 1978. Genetic studies of the *lacI* repressor. J Mol Biol 126:847–863.

Feron VJ, de Vrijer F, Woutersen RA, Cassee FR, van Bladeren PJ. 1991. Aldehydes: Occurrence, carcinogenic potential, mechanism of action and risk assesssment. Mutat Res 259: 363–385.

Fuscoe JC, Zimmerman LJ, Harrington-Brock K, Moore MM. 1992. Large deletions are tolerated at the *hprt* locus of in vivo derived human T lymphocytes. Mutat Res 283:255–262.

Gay P, Contamine D. 1993. Study of the ref(2)P locus of *Drosophila melongaster*: II. Genetic studies of the 37DF region. Mol Gen Genet 239:361–370.

Gibbs RA, Nguyen PN, Edwards A, Civitello AB, Caskey CT. 1990. Multiplex DNA deletion detection and exon sequencing of the hypoxanthine phosphoribosyltransferase gene in Lesch-Nyhan families. Genomics 7:235–244.

Goodrow T, Reynolds S, Maronpot R, Anderson M. 1990. Activation of K-ras by codon 13 mutations in C57BL/6 x C3HF1 mouse tumors induced by exposure to 1,3-butadiene. Cancer Res 50:4818–4823.

Gorelick NJ, Andrews JL, Gu M, Glickman BW. 1995. Mutational spectra in the lacI gene in skin from 7,12-dimethylbenz[a]anthracene–treated and untreated transgenic mice. Mol Carcinog 14:53–62.

Gossen JA, Martus HJ, Wei JY, Vijg J. 1995. Spontaneous and X-ray induced deletion mutations in a lacZ plasmid-based transgenic mouse model. Mutat Res 331:89–97.

Hayes RB, Xi L, Bechtold WE, Rothman N, Yao M, Henderson R, Zhang L, Smith MT, Zhang D, Wiemels J, Dosemeci M, Yin S, O'Neill JP. 1996. *hprt* Mutation frequency among workers exposed to 1,3-butadiene in China. Toxicology 113:100–105.

Heddle JA, Cimino MC, Hayashi M, Romagna F, Shelby MD, Tucker JD, Vanparys PH, MacGregor JT. 1991. Micronuclei as an index of cytogenetic damage: Past, present, and future. Environ Mol Mutagen 18:277–291.

Himmelstein MW, Acquavella JF, Recio L, Medinsky MA, Bond JA. 1997. Toxicology and epidemiology of 1,3-butadiene. CRC Crit Rev Toxicol 27:1–108.

Himmelstein MW, Asgharian B, Bond JA. 1995. High concentrations of butadiene epoxides in livers and lungs of mice compared to rats exposed to 1,3-butadiene. Toxicol Appl Pharmacol 132:281–288.

Himmelstein MW, Turner MJ, Asgharian B, Bond JA. 1994. Comparison of blood concentrations of 1,3-butadiene and butadiene epoxides in mice and rats exposed to 1,3-butadiene by inhalation. Carcinogenesis 15:1479–1486.

Huff JE, Melnick RL, Solleveld HA, Haseman JK, Powers M, Miller RA. 1985. Multiple organ carcinogenicity of 1,3-butadiene in $B6C3F_1$ mice after 60 weeks of inhalation exposure. Science 227:548–549.

International Agency for Research on Cancer. 1992. 1,3-Butadiene. In: Exposures to Mists and Vapours from Strong Inorganic Acids and other Industrial Chemicals, pp. 237–285. IARC, Lyon, France.

Jacobson-Kram D, Rosenthal SL. 1995. Molecular and genetic toxicology of 1,3-butadiene. Mutat Res 339:121–130.

Jauhar PP, Henika PR, MacGregor JT, Wehr CM, Shelby MD, Murphy SA, Margolin BH. 1988. 1,3-Butadiene: Induction of micronucleated erythrocytes in the peripheral blood of $B6C3F_1$ mice exposed by inhalation for 13 weeks. Mutat Res 209:171–176.

Jolly DJ, Okayama H, Berg P, Esty AC, Filpula D, Bohlen P, Johnson GG, Shively JE, Hunkapillar T, Friedman T. 1983. Isolation and characterization of a full length expressible cDNA for human hypoxanthine phosphoribosyltransfrerase. Proc Natl Acad Sci USA 80:477–481.

Kohler SW, Provost GS, Fieck A, Kretz PL, Bullock WO, Sorge JA, Putman DL, Short JM. 1991. Spectra of spontaneous and mutagen-induced mutations in the lacI gene in transgenic mice. Proc Natl Acad Sci USA 88:7958–7962.

Koivisto P, Kostiainen R, Kilpeläinen I, Steinby K, Peltonen K. 1995. Preparation characterization and ^{32}P-post-

labeling of butadiene monoepoxide adenine adducts. Carcinogenesis 16:2999–3007.

Koivisto P, Sorsa M, Pacchierotti F, Peltonen K. 1997. ^{32}P-Postlabeling/HPLC assay reveals an enantioselective adduct formation in N7 guanine residues in vivo after 1,3-butadiene inhalation exposure. Carcinogenesis 18:430–443.

Lähdetie J, Grawé J. 1997. Flow cytometric analysis of micronucleus induction in rat bone marrow polychromatic erythrocytes by 1,2,3,4-diepoxybutane, 3,4-epoxy-1-butene, and 1,2-epoxybutane-3,4-diol. Cytometry 28: 228–235.

Lawley PD, Brooks P. 1963. Further studies on the alkylation of nucleic acids and their constituent nucleotides. Biochem J 89:127.

Lawley PD, Brookes P. 1967. Interstrand cross-linking of DNA by difunctional alkylating agents. J Mol Biol 25:143–160.

Leavens TL, Farris GM, James RA, Shah R, Wong VA, Marshall MW, Bond JA. 1997. Genotoxicity and cytotoxicity in male B6C3F1 mice following exposures to mixtures of 1,3-butadiene and styrene. Environ Mol Mutagen 29:335–345.

Leuratti C, Jones NJ, Marafante E, Kostiainen R, Peltonen K, Waters R. 1994. DNA damage induced by the environmental carcinogen butadiene: Identification of a 1,2,3,4-diepoxybutane-adenine adduct and its detection by ^{32}P-postlabeling. Carcinogenesis 15:1903–1910.

Li CY, Yandell DW, Little JB. 1992. Molecular mechanisms of spontaneous and induced loss of heterozygosity in human cells in vitro. Somatic Cell Genet 18:77–87.

Lynch AM, Parry JM. 1993. The cytochalasin-B micronucleus/kinetochore assay in vitro: Studies with 10 suspected aneugens. Mutat Res 287:71–86.

Macaluso M, Larson R, Delzell E, Sathiakumar N, Hovinga M, Julian J, Muri DCF, Cole P. 1996. Leukemia and cumulative exposure to butadiene styrene and benzene among workers in the synthetic rubber industry. Toxicology 113:190–202.

Manjanatha MG, Chen JB, Shaddock JG Jr, Harris AJ, Shelton SD, Casciano DA. 1996. Molecular analysis of *lacI* mutations in Rat2 cells exposed to 7,12-dimethylbenz[*a*]anthracene: Evidence for DNA sequence and DNA strand biases for mutation. Mutat Res 372:53–64.

Medinsky MA, Leavens TL, Csanády GA, Gargas ML, Bond JA. 1994. In vivo metabolism of butadiene by mice and rats: A comparison of physiological model predictions and experimental data. Carcinogenesis 15:1329–1340.

Melnick RL, Huff J, Chou BJ, Miller RA. 1990a. Carcinogenicity of 1,3-butadiene in B6C3F$_1$ mice at low exposure concentrations. Cancer Res 50:6592–6599.

Melnick RL, Huff JE, Roycroft JH, Chou BJ, Miller RA. 1990b. Inhalation toxicology and carcinogenicity of 1,3-butadiene in B6C3F1 mice following 65 weeks of exposure. Environ Health Perspect 86:27–36.

Melnick RL, Kohn MC. 1995. Mechanistic data indicate that 1,3-butadiene is a human carcinogen. Carcinogenesis 16:157–163.

Meng Q, Recio L, Reilly AA, Wong BA, Bauer M, Walker VE. 1999. Comparison of the mutagenic potency of 1,3-butadiene at the hprt locus of T-lymphocytes following inhalation exposure of female B6C3F1 mice and F344 rats. Carcinogenesis 19:1019–1027.

Miller RG. 1980. Simultaneous Statistical Inference, 2nd ed. Springer-Verlag, New York, NY.

National Toxicology Program. 1984. Toxicology and carcinogenesis studies of 1,3-butadiene in B6C3F$_1$ mice (inhalation studies). Technical Report Number 228. National Toxicology Program, Research Triangle Park, NC.

Nauhaus SK, Fennell TR, Asgharian B, Bond JA, Sumner SC. 1996. Characterization of urinary metabolites from Sprague-Dawley rats and B6C3F1 mice exposed to [1,2,3,4-^{13}C]butadiene. Chem Res Toxicol 9:764–773.

Neagu I, Koivisto P, Neagu C, Kostianen R, Stenby K, Peltonen K. 1995. Butadiene monoxide and deoxyguanosine alkylation products at the N7-position. Carcinogenesis 16:1809–1813.

Nieusma JL, Claffey DJ, Maniglier-Poulet C, Imiolczyk T, Ross D, Ruth JA. 1997. Stereochemical aspects of 1,3-butadiene metabolism and toxicity in rat and mouse liver microsomes and freshly isolated rat hepatocytes. Chem Res Toxicol 10:450–456.

Occupational Safety and Health Administration. 1996. Occupational exposure to 1,3-butadiene: Final rule. Fed Regist 61:56745–56795.

Owen PE, Glaister JR. 1990. Inhalation toxicity and carcinogenicity of 1,3-butadiene in Sprague-Dawley rats. Environ Health Perspect 86:19–25.

Parent RA, Carevello HE, Harbell JW. 1991. Gene mutation assay of acrolein in the CHO/HGHPRT test system. J Appl Toxicol 11: 91–95.

Reardon JT, Liljestrand-Golden CA, Dusenberry RL, Smith PD. 1987. Molecular analysis of 1,2,3,4-diepoxybutane-

induced deletions at the rosy locus of *Drosophila melongaster*. Genetics 115:323–331.

Recio L, Meyer K. 1995. Increased frequency of mutations at A•T base pairs in the bone marrow of B6C3F$_1$ *lacI* transgenic mice exposed to 1,3-butadiene. Environ Mol Mutagen 26:1–8.

Recio L, Meyer KG, Pluta LJ, Moss OR, Saranko CJ. 1996. Assessment of 1,3-butadiene mutagenicity in the bone marrow of B6C3F$_1$ *lacI* transgenic mice (Big Blue™): A review of mutational spectrum and *lacI* MF after a 5-day 625 ppm 1,3-butadiene exposure. Environ Mol Mutagen 28:424–429.

Recio L, Osterman-Golkar S, Csanády GA, Turner MJ, Mhyr B, Moss O, Bond JA. 1992. Determination of mutagenicity in tissues of transgenic mice following exposure to 1,3-butadiene and *N*-ethyl-*N*-nitrosourea. Toxicol Appl Pharmacol 117:58–64.

Recio L, Pluta LJ, Meyer KG. 1998. The in vivo mutagenicity and mutational spectrum at the *lacI* transgene recovered from the spleens of B6C3F$_1$ mice following a 4-week inhalation exposure to 1,3-butadiene. Mutat Res 401(1–2):99–110.

Recio L, Steen A-M, Bond JA. 1997. Linking dosimetry of butadiene with mutational spectra to assess the roles of epoxybutane and diepoxybutane in the genotoxicity of butadiene. CIIT Activities 17(8):1–9.

Saranko CJ, Recio L. 1998. The butadiene metabolite, 1,2:3,4-diepoxybutane, induces micronuclei but is only weakly mutagenic at *lacI* in the Big Blue™ Rat2 *lacI* transgenic cell line. Environ Mol Mutagen 31(1):32–40.

Sasiadek M, Järentaus H, Sorsa M. 1991a. 1,3-Butadiene and its epoxides induce sister-chomatid in human lymphocytes in vitro. Mutat Res 261:117–121.

Sasiadek M, Järentaus H, Sorsa M. 1991b. Sister-chromatid exchanges induced by 1,3-butadiene and its epoxides in CHO cells. Mutat Res 263:47–50.

Seaton MJ, Follansbee MH, Bond JA. 1995. Oxidation of 1,2-epoxy-3-butene to 1,2:3,4-diepoxybutane by cDNA-expressed human cytochomes p450 2E1 and 3A4 and human mouse and rat liver microsomes. Carcinogenesis 16:2287–2293.

Selzer RR, Elfarra AA. 1996. Synthesis and biochemical characterization of N1-, N2-, and N7-guanosine adducts of butadiene monoxide. Chem Res Toxicol 9:126–132.

Selzer RR, Elfarra AA. 1997. Characterization of four N-3-thymidine adducts formed in vitro by the reaction of thymidine and butadiene monoxide. Carcinogenesis 18:193–198.

Sisk SC, Pluta L, Bond JA, Recio L. 1994. Molecular analysis of *lacI* mutants from bone marrow of B6C3F$_1$ transgenic mice following inhalation exposure of 1,3-butadiene. Carcinogenesis 15:471–477.

Sjöblom T, Lähdetie J. 1996. Micronuclei are induced in rat spermatids in vitro by 1,2,3,4-diepoxybutane but not by 1,2-epoxy-3-butene and 1,2-dihydroxy-3,4-epoxybutane. Mutagenesis 11:525–528.

Skopek TR, Liber HL, Penman BW, Thilly WG. 1978. Isolation of a human lymphoblastoid line heterozygous at the thymidine kinase locus: Possibility for a rapid human cell mutation assay. Biochem Biophys Res Commun 84:411–416.

Sorsa M, Peltonen K, Anderson D, Demopoulos NA, Neumann HG, Osterman-Golkar S. 1996. Assessment of environmental and occupational exposures to butadiene as a model for risk estimation of petrochemical emissions. Mutagenesis 11:9–17.

Steen A-M, Meyer KG, Recio L. 1997a. Analysis of *hprt* mutations occurring in human TK6 lymphoblastoid cells following exposure to 1,2,3,4-diepoxybutane. Mutagenesis 12:61–67.

Steen A-M, Meyer, KG, Recio L. 1997b. Characterization of *hprt* mutations following 1,2-epoxy-3-butene exposure of human TK6 cells. Mutagenesis 12:359–364.

Suri A, deBoer J, Kusser W, Glickman BW. 1996. A 3 milliTesla 60 Hz magnetic field is neither mutagenic nor comutagenic in the presence of menadione and MNU in a transgenic rat cell line. Mutat Res 372:23–31.

Suzuki T, Hayashi M, Sofuni T, Myhr BC. 1993. The concomitant detection of gene mutation and micronucleus induction by mitomycin C in vivo using *lacZ* transgenic mice. Mutat Res 285:219–224.

Tao KS, Urlando C, Heddle JA. 1993. Comparison of somatic mutation in a transgenic versus host locus. Proc Natl Acad Sci USA 90:10681–10685.

Tates AD, van Dam FJ, de Zwart FA, van Teylingen CM, Natarajan AT. 1994. Development of a cloning assay with high cloning efficiency to detect induction of 6-thioguanine–resistant lymphocytes in spleen of adult mice following in vivo inhalation exposure to 1,3-butadiene. Mutat Res 309:299–306.

Thornton-Manning JR, Dahl AR, Bechtold WE, Henderson RF. 1996. Gender and species differences in the metabolism of 1,3-butadiene to butadiene monoepoxide and butadiene diepoxide in rodents following low-level inhalation exposures. Toxicology 113:322–325.

Tice RR, Boucher R, Luke CA, Shelby MD. 1987. Comparative cytogenetic analysis of bone marrow damage induced in male B6C3F$_1$ mice by multiple exposures to gaseous 1,3-butadiene. Environ Mutagen 9:235–250.

Tretyakova NY, Lin YP, Upton PB, Sangaiah R, Swenberg JA. 1996. Macromolecular adducts of butadiene. Toxicology 113:70–76.

Tretyakova N, Lin Y, Sangaiah R, Upton PB, Swenberg JA. 1997. Identification and quantitation of DNA adducts from calf thymus DNA exposed to 3,4-epoxy-1-butene. Carcinogenesis 18:137–147.

U.S. Environmental Protection Agency. 1991. Preliminary Draft List of Categories and Subcategories Under Section 112 of the Clean Air Act. Office of Air Quality Planning and Standards, Research Triangle Park, NC. Fed Regist 56:28548–28557.

Vrieling H, Venema J, van Rooyen ML, van Hoffen A, Menichini P, Zdzienicka MZ, Simons JWIM, Mullenders LHF, van Zeeland AA. 1991. Strand specificity for UV-induced DNA repair and mutation in the Chinese hamster HPRT gene. Nucleic Acids Res 19(9):2411–2415.

Ward JB Jr, Ammenheuser MM, Bechtold WE, Whorton EB Jr, Legator MS. 1994. *hprt* Mutant lymphocyte frequencies in workers at a 1,3-butadiene production plant. Environ Health Perspect 102:79–85.

Ward JB Jr, Ammenheuser MM, Whorton EB Jr, Bechtold WE, Kelsey KT, Legator MS. 1996. Biological monitoring for mutagenic effects of occupational exposure to butadiene. Toxicology 113:84–90.

Wiseman RW, Cochan C, Dietrich W, Lander ES, Söderkvist P. 1994. Allelotyping of butadiene-induced lung and mammary adenocarcinomas of B6C3F$_1$ mice: Frequent losses of heterozygosity in regions homologous to human tumor-suppressor genes. Proc Natl Acad Sci USA 91:3759–3763.

Wyborski DL, Malkhosyan S, Moores J, Perucho M, Short JM. 1995. Development of a rat cell line containing stably integrated copies of a lambda/*lacI* shuttle vector. Mutat Res 334:161–165.

Xi L, Zhang L, Wang Y, Smith MT. 1997. Induction of chromosome-specific aneuploidy and micronuclei in human lymphocytes by metabolities of 1,3-butadiene. Carcinogenesis 18:1687–1693.

Xiao Y, Tates AD. 1995. Clastogenic effects of 1,3-butadiene and its metabolites 1,2-epoxy-3-butene and 1,2,3,4-diepoxybutane in splenocytes and germ cells of rats and mice in vivo. Environ Mol Mutagen 26(2):97–108.

Zhuang SM, Cochran C, Goodrow T, Wiseman RW, Söderkvist P. 1997. Genetic alterations of *p53* and *ras* genes in 1,3-butadiene– and 2′,3′-dideoxycytidine–induced lymphomas. Cancer Res 57:2710–2714.

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Ann-Marie Steen received a D.M.Sc. degree in medical genetics and genetic toxicology in 1993 from the Karolinska Institute in Stockholm, Sweden. Her doctoral research involved work on gene expression and mutation at the *hprt* locus in human cells. As a postdoctoral fellow at CIIT from 1994 to 1996, Dr. Steen studied the mutational spectra of butadiene and its metabolites and the in vivo mutagenesis of ethylene oxide in the laboratory of Dr. Leslie Recio. Her field of interest is the molecular analysis of gene expression and mutations. Dr. Steen is currently a research scientist in safety assessment at Astra AB in Sweden.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Steen A-M, Recio L. 1995. Determination of the mutagenicity and mutational spectrum in human TK6 cells of 1,2,3,4-diepoxybutane, a genotoxic metabolite of 1,3-butadiene. In: Abstracts, October 20, 1995, GEMS Meeting.

Saranko CJ, Recio L. 1996. Lack of detectable mutagenicity at the *lacI* transgene in Big Blue(R) rat embryo fibroblasts following exposure to 1,2,3,4-diepoxybutane (abstract). Fundam Appl Toxicol Suppl 30(1), Part 2:234.

Steen A-M, Meyer K, Recio L. 1996. Mutational spectra at *hprt* for two epoxide metabolites of 1,3-butadiene in human TK6 lymphoblastoid cells (abstract). In: Annual Meeting of the American Association for Cancer Research, April 20–24, 1996, Washington, DC. Proc Am Assoc Cancer Res 37:142.

Steen A-M, Meyer K, Recio L. 1996. Mutational spectrum of 1,2,3,4-diepoxybutane in human TK6 lymphoblastoid cells (abstract). Environ Mol Mutagen 27(Suppl):65.

Recio L, Henderson R, Meyer K, Pluta L, Saranko CJ, Steen A-M. 1997. In vivo and in vitro mutagenicity and mutational spectrum of the butadiene metabolites 1,2-epoxy-3-butene and 1,2,3,4-diepoxybutane (abstract). In: Cancer and Genetic Risk Assessment: Low Dose–Effect Studies, Satellite Meeting, September 4–6, 1997. DKFZ, Heidelberg, Germany.

Recio L, Henderson R, Meyer K, Pluta L, Saranko CJ, Steen A-M. 1997. In vivo and in vitro mutagenicity and mutational spectrum of the butadiene metabolites 1,2-epoxy-3-butene and 1,2,3,4-diepoxybutane to assess their roles in the in vivo mutagenicity of 1,3-butadiene (abstract). Mutat Res 379(Suppl 1):S148.

Saranko CJ, Recio L. 1997. The butadiene metabolite, 1,2:3,4-diepoxybutane, induces micronuclei but is only weakly mutagenic at *lacI* in the Big Blue Rat2 *lacI* transgenic cell line. Environ Mol Mutagen 31(1):32–40.

Saranko CJ, Recio L, Henderson RF. 1997. In vitro and in vivo mutagenicity at the *lacI* transgene and induction of micronuclei by 1,2,3,4-diepoxybutane (abstract). Fundam Appl Toxicol 36(1), Part 2:308.

Steen A-M, Meyer KG, Recio L. 1997. Analysis of *hprt* mutations occurring in human TK6 lymphoblastoid cells following exposure to 1,2,3,4-diepoxybutane. Mutagenesis 12(2):61–67.

Steen A-M, Meyer KG, Recio L. 1997. Characterization of *hprt* mutations following 1,2-epoxy-3-butene exposure of human TK6 cells. Mutagenesis 12(5):359–364.

Saranko CJ, Pluta LJ, Recio L. 1998. Molecular analysis of *lacI* mutants from transgenic fibrobasts exposed to the butadiene metabolite 1,2-epoxybutene. Carcinogenesis 19(11):1879–1887.

ABBREVIATIONS AND OTHER TERMS

ANOVA	analysis of variance
BD	1,3-butadiene
BDO	1,2-epoxy-3-butene
BDO_2	1,2,3,4-diepoxybutane
BDO-diol	1,2-dihydroxy-3,4-epoxybutane
bp	base pair or pairs
CYP2E1	cytochome P450 2E1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methane sulfonate
ENU	*N*-ethyl-*N*-nitrosourea
hprt	hypoxanthine phosphoribosyltransferase gene
IARC	International Agency for Research on Cancer
lacI mice	$B6C3F_1$ *lacI* transgenic mice
lacI rats	F344 *lacI* transgenic rats
MF	mutant frequency
NMR	nuclear magnetic resonance
OSHA	Office of Safety and Health Administration
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Rat2 cells	Rat2 *lacI* transgenic fibroblasts
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
6-TG	6-thioguanine
tk	thymidine kinase gene
TK6 cells	human TK6 lymphoblastoid cells
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

INVESTIGATORS' REPORT

1,3-Butadiene: Cancer, Mutations, and Adducts

Part III: In Vivo Mutation of the Endogenous *hprt* Genes of Mice and Rats by 1,3-Butadiene and Its Metabolites

Vernon E. Walker and Quanxin Meng

ABSTRACT

1,3-Butadiene (BD)*, an important chemical used mainly in the production of synthetic rubber, is a potent carcinogen in mice, a weak carcinogen in rats, and a suspected carcinogen in humans. To provide a better understanding of the mutagenic mechanisms involved in interspecies differences in BD-induced carcinogenesis, studies were conducted in rodents to test two hypotheses: (a) the mutagenic potency of BD at the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) locus of T lymphocytes (T cells) can be used to quantify interspecies differences in BD-induced carcinogenicity in exposed rodents and (b) comparison of the mutagenic potency and specificity of BD and racemic mixtures of two epoxy metabolites, 1,2-epoxy-3-butene (BDO) and 1,2,3,4-diepoxybutane (BDO_2), at the *hprt* locus of T cells can be used to define the relative contribution of each intermediate to observed BD mutagenicity in each species. The first hypothesis was investigated by determining the effects of exposure duration and elapsed time after exposures on *hprt* mutant frequencies (MFs) in T cells from thymus and spleen of female $B6C3F_1$ mice and F344 rats (4 to 5 weeks old). In this study, rodents were exposed by inhalation to 0 or 1,250 parts per million (ppm) BD for up to 2 weeks, or to 0 or 625 ppm BD for up to 4 weeks (with all exposures 6 hours/day, 5 days/week). The second hypothesis was examined by defining the effects of exposure concentration and elapsed time after exposures on the *hprt* MFs in splenic T cells from mice and rats exposed by inhalation to BD (0, 20, 62.5, or 625 ppm), BDO (0, 2.5, or 25 ppm), or BDO_2 (0, 2, or 4 ppm) for 4 weeks (all exposures 6 hours/day, 5 days/week). The *hprt* MFs were measured weekly or biweekly using the T cell cloning assay for up to 10 weeks after the last exposure.

The mutagenic potency of BD (represented by the difference in the areas under the mutant T cell "manifestation" curves [or the "change in MFs over time"] of exposed versus control animals) was significantly greater in mice (4.4-fold) than in rats following 2 weeks of exposure to 1,250 ppm BD. Mutagenic potency in mice was 8.5-fold greater than that in rats following 4 weeks of exposure to 625 ppm BD. These *hprt* MF data provide the first evidence that BD is mutagenic in the rat, albeit the mutagenic response was significantly less than that observed in similarly exposed mice. In addition, the MF data from the two exposure-duration studies indicate that both exposure concentration and exposure duration are important in determining the magnitude of the mutagenic response to BD. The relative contribution of BDO versus BDO_2 to overall BD mutagenicity was evaluated by exposing mice and rats to carefully chosen concentrations of BD and racemic mixtures of BDO and BDO_2 (that is, 62.5, 2.5, and 4.0 ppm, respectively) and comparing the mutagenic potency of each compound when comparable blood levels of metabolites were achieved. The resulting MF data indicate that (±)-BDO_2 is a major contributor to the mutagenicity of BD in mice at lower BD exposure levels (≤ 62.5 ppm), whereas other metabolites and stereochemical configurations are responsible for mutations in BD-exposed rats and for the incremental mutagenic effects at higher exposure levels in mice. Molecular analysis of *hprt* cDNA from expanded T cell clones from control and BD-exposed mice demonstrated an increased frequency of large deletions in exposed animals ($p = 0.016$), presumably associated with in situ formation of (±)-BDO_2, *meso*-BDO_2, or both. Results of these mutagenicity experiments, along with data from collaborative studies of DNA adducts from the same animals,

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

This Investigators' Report is Part III of Health Effects Institute Research Report Number 92, which also includes four additional Investigators' Reports (Parts I, II, IV, and V) by different research groups, a Commentary by the Health Review Committee about Parts II and III, a Commentary on Part I, a Commentary on Parts IV and V, and an HEI Statement about the five butadiene research projects reported here. Correspondence regarding the Part III Investigators' Report may be addressed to Dr. Vernon E. Walker, Laboratory of Human Toxicology and Molecular Epidemiology, Wadsworth Center, New York State Department of Health, Albany, NY 12201-0509.

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should provide a better understanding of the interspecies variation in carcinogenic response to BD and improve the extrapolation of rodent data to the estimation of cancer risk in exposed persons.

INTRODUCTION

1,3-Butadiene, an important chemical used in the production of synthetic rubber, is a known carcinogen in rodents and a suspected carcinogen in occupationally exposed humans. Long-term animal studies have demonstrated that BD is a multisite carcinogen in $B6C3F_1$ mice and in Sprague-Dawley rats. However, the spectrum of BD-induced tumor types is considerably different in each species, and the cancer potency is much greater in the mouse than in the rat (Melnick and Kohn 1995). Target tissues for BD-induced carcinogenesis include liver, mammary gland, and ovary in female mice; forestomach, Harderian gland, heart (hemangiosarcoma), lung, and lymphatic system in both female and male mice; mammary gland, thyroid gland, and uterus in female rats; and pancreas and testis in male rats (see review of relative frequency of specific tumor types in Himmelstein et al. 1997). In contrast, epidemiologic studies evaluating the effects of BD exposures have not been conclusive as a whole, but rather they have produced limited evidence that lymphatic and hematopoietic cancers are possible responses to BD exposure in humans (Landrigan 1993; Bond et al. 1995). This variation in carcinogenic response to BD in different species makes it difficult to extrapolate rodent data to humans and thus to assess potential health hazards to persons exposed to BD in the occupational environment. Because BD exposures have been associated with apparently increased incidences of lymphosarcomas and leukemias in some human studies (Downs et al. 1987; Santos-Burgoa et al. 1992; Divine et al. 1993; Delzell et al. 1996; Divine and Hartman 1996) and have produced increased incidences of thymic lymphomas and leukemias in mice, it is important to elucidate the mutagenic and carcinogenic mechanisms of chemically induced leukemogenesis in both humans and mice. Elucidation may enable us to evaluate relevant biological markers that would improve risk extrapolation between BD-exposed rodents and humans.

The genotoxic potential of BD has been demonstrated in both rodents and humans, although the species susceptibility to perturbations in various biological markers of DNA damage differs greatly (reviewed in Jacobson-Kram and Rosenthal 1995, and Himmelstein et al. 1997). For example, BD causes increased frequencies of chromosomal aberrations, sister chromatid exchanges, and micronuclei in exposed mice, whereas these increases have not been observed in similarly treated rats (Cunningham et al. 1986). In persons occupationally exposed to BD, documentation of exposure-related effects for these three cytogenetic endpoints (Sorsa et al. 1994; Tates et al. 1996) has been lacking except for two recent reports of increased frequencies of chromosomal aberrations in exposed groups (Sorsa et al. 1996; Šrám et al. 1998). Results of the study by Sorsa and colleagues (1996) suggested that the polymorphism in the glutathione *S*–transferase *T1* (*GSTT1*) gene might play a role in determining interindividual sensitivity to BD-induced chromosomal aberrations. The percentage of cells with chromosomal aberrations was significantly increased among exposed workers lacking the *GSTT1* gene as compared with the percentage in exposed workers with the gene. The other polymorphic gene, *GSTM1*, showed no association in chromosomal aberrations or other cytogenetic endpoints. Also, BD has been reported to produce increased frequencies of mutations or mutant variants at the *hprt* locus in T lymphocytes of both exposed mice (Cochrane and Skopek 1994b; Tates et al. 1994) and humans (Ward et al. 1994, 1996a,b), as well as at the *lacI* transgene in bone marrow of exposed $B6C3F_1$ transgenic mice (Sisk et al. 1994; Recio et al. 1996a,b). Furthermore, the increased frequencies of *hprt* variants in humans, as measured by the autoradiography assay, were associated in a dose-related manner with a specific BD metabolite found in urine in two different populations of workers (Ward et al. 1994, 1996a,b). These observations in humans exposed to BD at levels typical of an industrial setting (< 3 ppm) are of particular interest because BD concentrations as low as 6.25 ppm increase the incidence of genetic damage and the incidence of lung tumors in mice. These genotoxicity data, along with the currently available carcinogenicity, epidemiologic, and mechanistic data, have recently been used to support opposing points of view that BD is (Melnick and Kohn 1995) or is not (Bond et al. 1995) likely to be carcinogenic to humans at exposure levels encountered in the general or occupational environment.

Although susceptibility to various types of DNA damage appears to differ greatly among mice, rats, and individual persons, the genotoxicity data for BD strongly suggest that the mutagenicity and carcinogenicity of this chemical are related to its metabolic activation to several DNA-reactive intermediates including BDO, BDO_2, and 1,2-dihydroxy-3,4-epoxybutane (BDO-diol) (reviewed in Himmelstein et al. 1997). Two of these three metabolites (that is, BDO and BDO_2) have been measured in tissues of BD-exposed mice and rats, and the greater cancer potency in the mouse has been attributed in part to higher circulating levels of these genotoxic intermediates compared with the circulating levels of intermediates in rats exposed to the same levels of

parent compound (Himmelstein et al. 1995, 1996; Thornton-Manning et al. 1995a). Still, the relative contributions of BDO and BDO_2 to the mutagenic and carcinogenic actions of BD in mice and rats remains unclear (Bond et al. 1995; Melnick and Kohn 1995). Thus, in vivo mutagenicity studies of BD and its metabolites in "reporter" genes of similarly exposed mice and rats provide a potential means of further clarifying species differences in carcinogenicity. For example, several investigations have shown that BD is mutagenic at both the endogenous *hprt* gene (Cochrane and Skopek 1994b; Tates et al. 1994) and *lacI* transgene of mice (Sisk et al. 1994), but additional studies are needed to prove the premise that BD is also mutagenic in the rat.

The role of stereochemistry in species-dependent metabolism and toxicity of BD is an additional complicating factor that must be considered in clarifying species differences in BD-induced mutagenesis and carcinogenesis. Stereochemical considerations arise following the initial oxidation of BD to BDO because a stereocenter is generated, and the chirality of this metabolite may affect both the subsequent biotransformation pathways (Nieusma et al. 1997) and the formation and persistence of BDO-induced DNA adducts (Koc et al. 1999). For example, in vitro studies with mouse- and rat-liver microsomes have demonstrated species differences in both the production of BDO and the relative proportions of (*R*)-BDO and (*S*)-BDO (Westuba et al. 1989; Nieusma et al. 1997). Reasons for both a different enantiomer ratio in mice compared with the ratio in rats and for the change in ratio over time in the rat (Nieusma et al. 1997) are not known, but species differences may exist in the interaction of BD with various cytochrome P450s (Himmelstein et al. 1997) and in the hydrolysis of the enantiomers. BDO has been shown to be further oxidized in microsomes of human, rat, and mouse liver to a mixture of (±)-BDO_2 and *meso*-BDO_2, with the relative amounts of each diastereomer dependent upon the concentration of BDO applied (Elfarra et al. 1996; Krause and Elfarra 1997). *meso*-BDO_2 appears to be more cytotoxic in vitro than does the racemic mixture of enantiomers (Nieusma et al. 1997), but it is more readily hydrolyzed in human- and rat-liver microsomes compared with hydrolysis in mouse-liver microsomes (Krause and Elfarra 1997). These findings demonstrate the potential importance of stereochemistry in BD biotransformation and cytoxicity, and they emphasize the need for studies investigating the contributions of specific stereochemical configurations to the mutagenicity of BD in rodent models.

The three original purposes of the current project were (a) to determine if exposures to BD produce increased MFs at the *hprt* locus of T cells from exposed rats, (b) to determine if the MFs at *hprt* of T cells from rats and mice exposed to BD can be correlated with species differences in susceptibility to cancer, and (c) to determine if the mutagenic potency and *hprt* exon 3 mutational spectra data from mice and rats exposed either to BD or directly to its individual epoxy metabolites can reveal which DNA-reactive intermediates are responsible for mutations in each species. To this end, the T cell cloning and sequencing assay (Jones et al. 1987b; Aidoo et al. 1991; Skopek et al. 1992; Mittelstaedt et al. 1995) was used for determining the frequency and nature of mutations at the *hprt* locus of T lymphocytes isolated from spleen and thymus of control and chemically exposed rodents. T cells were selected as a mutational target partly because BD produces elevated incidences of thymic lymphomas and T cell leukemias in mice, although not in rats (Huff et al. 1985; Owen et al. 1987; Melnick et al. 1990). Experiments with BD metabolites were restricted to racemic mixtures of BDO and BDO_2 in large part because in vitro mutagenicity studies in human lymphoblastoid cells demonstrated that the mutagenic efficiencies of these two racemic mixtures were greater than that of BDO-diol (Cochrane and Skopek 1994a). These experiments seemed to be a logical starting point for systematic investigation of the role of epoxy metabolites and specific stereochemical configurations in BD-induced mutations at the *hprt* locus of mice and rats.

The approach used to estimate the mutagenic potency of BD, (±)-BDO, and (±)-BDO_2 in splenic T cells from exposed rodents is based on a method with two components, one biological and the other mathematical. The biological component is based upon the hypothesis that the time dependence of the frequency of *hprt* mutant T cells in the spleen of rodents is largely due to the renewal of peripheral T lymphocyte populations; that is, that the mutations are induced primarily in T cells in bone marrow and thymus but are expressed as mutant cells that migrate to the spleen and other peripheral tissues (Jones et al. 1987b; Walker and Skopek 1993b). Because increasing age apparently leads to a reduction in the rate of renewal of peripheral lymphocyte populations (Hirokawa and Makinodan 1975) and to a decline in the number of T cells migrating from the thymus to the periphery (Sollay et al. 1980), mice and rats of nearly identical age were exposed by inhalation either to chamber supply air, BD, (±)-BDO, or (±)-BDO_2. A novel mathematical approach was used to derive an estimate of total mutagenic burden for interspecies comparison by integrating the area under the mutant T cell "manifestation" curves (Heddle 1998) for treated versus control mice or rats. In the case of splenic T lymphocytes, the mutant manifestation curve or the change in MFs over time following exposure of mice and rats to alkylating agents encompasses the

period of time required for T cell replication and mutant fixation, expression of the *hprt* mutant phenotype and dilution of the wild-type HPRT protein, migration of mutant T cells from the thymus to peripheral tissues, and finally, selective removal of *hprt* mutant T cells from the spleen and peripheral blood.

During the course of this project, additional experiments were performed to evaluate the effects of several growth factors and culture medium supplements on the in vitro growth of T lymphocytes isolated from mouse spleen. These experiments arose from the need to reduce the impediments in mutagenicity studies caused by the relatively poor growth potential of mouse T cells in vitro; poor growth leads to low cloning efficiencies and limited expansion of *hprt* mutant clones for molecular analyses of mutations requiring mRNA or clonally pure genomic DNA. During experiments to evaluate the mutagenic potency of BD in the mouse, cloning efficiencies for mouse T cells were improved dramatically, and a subset of *hprt* mutant clones from control and BD-exposed mice were used in pilot studies that led to the development of a procedure for large-scale expansion of mouse T cell clones (Meng et al. 1998b). Unfortunately, the methods for expanding mouse and rat T cell clones were not perfected in time to propagate *hprt* mutant clones collected from rats exposed to BD or from rats and mice exposed to (±)-BDO or (±)-BDO_2 as part of the mutagenic potency studies described here. Notwithstanding, a set of over 100 expanded mutant T cell clones from control and BD-exposed mice were successfully collected and molecular analyses were performed to compare mutations occurring over the entire *hprt* gene.

SPECIFIC AIMS

The major hypotheses of this project were that (a) BD is mutagenic in the rat as well as in the mouse, (b) the mutagenic potency of BD at the *hprt* locus of T cells can be used qualitatively or quantitatively to indicate species differences in BD-induced carcinogenicity, and (c) comparisons of the mutagenic potency and specificity of BD versus its epoxy metabolites (specifically, racemic mixtures of BDO and BDO_2) at the *hprt* locus of T cells can be used to define the relative role of racemic mixtures of BDO and BDO_2 in BD-induced mutagenesis in each species. These hypotheses were tested by completing the following four specific aims:

1. to determine the species differences in the mutagenic potency of BD by evaluating the effects of (a) exposure duration and (b) elapsed time after exposure on the frequency of *hprt* mutations in T cells from exposed mice and rats versus the MF in T cells from control animals;
2. to determine the relative contribution of racemic mixtures of BDO and BDO_2 to BD-induced mutagenicity by evaluating the effects of (a) exposure duration, (b) elapsed time after exposure, and (c) exposure concentration on the frequency of *hprt* mutations in T cells from mice and rats exposed directly to the epoxy metabolites versus T cells exposed to the parent compound;
3. to characterize and compare the nature of mutations occurring in the exon 3 region of the *hprt* gene of T cells from mice and rats exposed to BD or racemic mixtures of BDO and BDO_2; and
4. to define and compare mutations occurring over the *hprt* gene of T cells from control and BD-exposed mice.

METHODS AND STUDY DESIGN

ANIMALS AND HUSBANDRY

Female $B6C3F_1$ mice and F344 rats that were 3 weeks (± 3 days) of age were purchased from Charles River Breeding Laboratories in Raleigh, NC (mice), Portage, MI (mice), or Holister, CA (rats), and were acclimated for about 10 days before initiation of inhalation exposures to BD, (±)-BDO, or (±)-BDO_2. All animals were free of virus titers as determined by standard mouse and rat virus antibody assays (Microbiological Associates, Bethesda, MD). The housing of rodents conformed to NIH guidelines (NIH Publication No. 86-23, 1985). Animals were randomly separated into air control and exposure groups by weight and were housed individually in hanging stainless-steel wire cages in rooms controlled for temperature (72 ± 4°F) and humidity (50 ± 10%) and regulated by a 12-hour light/dark cycle. All procedures involving the use of animals were approved by the Institutional Animal Care and Use Committees at the facilities where the exposures and animal experiments were performed.

CHEMICALS

The chemicals BD (99+% pure) and (±)-BDO were acquired from Aldrich Chemical Co. (Milwaukee, WI and St. Louis, MO). The purity of BDO was 97.4% on the basis of on-site nuclear magnetic resonance (NMR) analysis. Several lots of (±)-BDO_2 obtained from Aldrich Chemical Co. (St. Louis, MO) and Lancaster Synthesis, Inc. (Windham, NH) were combined and redistilled to obtain a purity of 99.2% by NMR analysis. [Hereafter in this report, BDO and BDO_2

will refer to the racemic mixtures of each metabolite except for specific references to (±)-BDO_2 and *meso*-BDO_2 in the Discussion and Conclusions section.] Reagents for cell culture were purchased from the following sources: 6-thioguanine and β-mercaptoethanol from Sigma Chemical Co. (St Louis, MO); concanavalin A (catalog #13821, lot #73910) from Worthington Biochemical Corp. (Freehold, NY); Lympholyte® M and R from Accurate Chemical and Scientific Corp. (Westbury, NY); HEPES buffer from Fisher Scientific (New York, NY); HL-1 from Hycor Biomedical, Inc. (Irvine, CA) and Biowhittaker, Inc. (Walkersville, MD); and rat T-STIM™ culture supplement and mouse recombinant interleukin-2 (IL-2) from Becton Dickinson Labware (Bedford, MA). Other medium components, including RPMI 1640 medium, fetal bovine serum, L-glutamine, nonessential amino acids, penicillin-streptomycin, and sodium pyruvate were obtained from Media and Glassware Support Service Group in the Wadsworth Center, New York State Department of Health (Albany, NY). Other chemicals or reagents for DNA extractions, polymerase chain reaction (PCR) amplifications, evaluation of PCR efficiency, performance of denaturing gradient gel electrophoresis (DGGE), and DNA sequencing were of the highest grade available from commercial sources and have been listed elsewhere (Skopek et al. 1992; Walker and Skopek 1993a).

SPECIFIC AIM 1

Animal Exposures

Experiments were conducted with BD to evaluate the effects of both exposure duration and elapsed time after inhalation exposures on the frequency of *hprt* mutant T lymphocytes in female $B6C3F_1$ mice and female F344 rats. The design of these experiments was drawn in part from earlier studies with BD or other alkylating agents. The $B6C3F_1$ mouse was selected as a model because tumor data are available for this strain from the National Toxicology Program's cancer bioassays of BD (NTP 1984; Huff et al. 1985; Melnick et al. 1990); in addition, transgenic mice of $B6C3F_1$ origin have been used to investigate the mutagenicity of inhaled BD at the *lacI* transgene (Sisk et al. 1994; Recio and Myer 1995; Recio et al. 1996a,b). Furthermore, the $B6C3F_1$ mouse and F344 rat were used in the development of the rodent T cell cloning and sequencing assays for *hprt* mutations (Aidoo et al. 1991; Skopek et al. 1992; Walker and Skopek 1993a; Mittelstaedt et al. 1995), and these models have been used to evaluate the mutagenicity of several chemicals, including BD and its major epoxy metabolites, in preweanling $B6C3F_1$ mice (Cochrane and Skopek 1994b). Female mice (and the corresponding sex of rats) were specifically selected for exposure to BD and its epoxy metabolites because they are more susceptible than male mice to the induction of thymic lymphomas and leukemias by BD (reviewed in Himmelstein et al. 1997) and several other epoxides or epoxy-forming chemicals (for example, ethylene oxide, acrylamide, and glycidol). The exposure concentration (1,250 ppm BD) and duration (2 weeks) were chosen for this work as a best effort to ensure that mutagenic effects would be demonstrated in both mice and rats. The exposure concentration of 1,250 ppm was the same as the high-dose level used in the first mouse bioassay (compared with 1,000 and 8,000 ppm BD used in a bioassay in Sprague-Dawley rats [Owen et al. 1987]), and the 2-week exposure duration was based upon earlier studies demonstrating mutagenic effects at the *lacI* and *hprt* loci of exposed transgenic (Sisk et al. 1994) and nontransgenic preweanling mice (Cochrane and Skopek 1994b), respectively.

Animal exposures were to the whole body in stainless-steel and glass 8-m^3 chambers to nominal 1,250 ppm BD, with chamber concentrations monitored at least once per hour using a Foxboro (Foxboro, MA) Wilkes 1A infrared detector. Control animals were exposed to chamber supply air with 0 ppm BD in a sham exposure. The animals were housed within exposure chambers throughout the experiment, and they had free access to food (NIH-07 certified feed) and water except during the 6-hour exposure periods when food was removed.

BD-exposed and control mice and rats were killed at specific postexposure times by CO_2 asphyxiation followed by exsanguination via cardiac puncture; the thymus, spleen, or both were removed aseptically for isolation of T cells for *hprt* mutation studies. As part of additional collaborative studies, heparinized blood and other target and nontarget tissues for BD-induced carcinogenesis were collected for measurements of hemoglobin and DNA adducts within the same animals.

Effects of Exposure Duration on *hprt* Mutant Frequencies in T Cells from Thymus of Mice and Rats Exposed to BD (Experiment 1)

To evaluate differences between mice and rats in the accumulation of *hprt* mutations in thymus over the course of a 2-week exposure to BD, groups of animals were exposed to 0 or 1,250 ppm (6 hours/day, 5 days/week) and then necropsied 2 weeks after a 1-week exposure or 2 weeks after a 2-week exposure for isolation of thymic T cells and measurement of MFs. Control (n = 4–8/group) and exposed (n = 10/group) mice and rats were 4 to 5 weeks of age at the beginning of exposure. Background MFs in T cells isolated from thymus of 4- to 5-week-old animals were established by determining the average values in groups of unexposed

mice and rats necropsied immediately prior to the first BD exposure (see Figure 1 in the Results section).

Effects of Elapsed Time After Exposure on *hprt* Mutant Frequencies in T Cells from Thymus and Spleen of Mice and Rats Exposed to BD (Experiment 2)

To evaluate differences between mice and rats in the changes of *hprt* MF in T cells from thymus and spleen following BD exposures, animals were exposed to 0 or 1,250 ppm BD for 2 weeks (6 hours/day, 5 days/week); groups of control and exposed animals were necropsied immediately after the last exposure and at additional time points up to 10 weeks after the last exposure (see Figures 2 and 3 in the Results section) for isolation of T cells and measurement of MFs. The control animals in this experiment included mice and rats necropsied before initial exposure, and mice and rats exposed to 0 ppm BD for 1 or 2 weeks and then necropsied at the same points after the last exposure as the analogous BD-exposed animals.

Isolation and Culture of *hprt* Mutant T Cells

The procedures for isolating T lymphocytes from thymus (Experiments 1 and 2) and spleen (Experiment 2) and for culturing *hprt* mutant T cell clones have been described in detail (Skopek et al. 1992; Walker and Skopek 1993a). Briefly, T cells were isolated by macerating thymus and spleen individually in medium, washing, suspending the cells in medium, and then layering the cells on a Ficoll gradient (Lympholyte® M for mice, Lympholyte® R for rats) for collection, washing, and resuspension in supplemented medium. Isolated T cells were primed overnight in RPMI 1640 medium supplemented with the mitogen concanavalin A (with the supplemented medium considered the "priming medium"), enumerated using a Coulter counter, and cultured in medium supplemented with growth factor (IL-2) and selective agent (6-thioguanine) in 96-well microtiter plates. To determine cloning efficiencies for T cells from each tissue, aliquots of samples were diluted in "cloning medium" (that is, complete medium lacking concanavalin A) so that there were 4 rat splenic lymphocytes/well, 8 mouse splenic lymphocytes/well, or 16 mouse or rat thymic lymphocytes/well cultured in the presence of lethally irradiated rat spleen lymphocytes ("feeder cells"). Excess lymphocytes isolated from control rats were used as a source of feeder cells. To isolate *hprt* mutants, primed cultures were diluted (to 5×10^5 cells/mL for mouse and rat thymic lymphocytes, 4×10^5 cells/mL for mouse splenic lymphocytes, and 5×10^5 cells/mL for rat splenic lymphocytes) with cloning medium supplemented with 1 µg 6-thioguanine/mL and then seeded for incubation in 96-well plates at 100 µL/well. Plates were scored for clonal colony growth at magnification of × 40, and confirmed at higher magnifications as necessary, on days 10 to 12. The *hprt* MF was calculated as described previously (Skopek et al. 1992). The MF data for individual control and BD-exposed groups at different time points were expressed as means ± SE, and MF data for all control animals (that is, preexposure and sham-exposed groups combined) were presented as means ± SD. The *hprt* mutant colonies from spleen of control and BD-exposed animals were collected and frozen for molecular analysis (see Specific Aim 3).

SPECIFIC AIM 2

Animal Exposures

The duration of exposures in the experiments for Specific Aim 2 were extended from those for Specific Aim 1 for two reasons. First, because experimental data for Specific Aim 1 suggested that the species difference in mutagenic response to BD would become more pronounced with longer exposures (that is, beyond 2 weeks) (see Results, Specific Aim 1), an additional experiment was conducted to evaluate the effects of exposure duration on *hprt* MFs in mice and rats exposed to BD for 2 and 4 weeks. Second, the exposure durations for the experiments assessing response to BDO and BDO_2 were set at 4 weeks as a best effort to ensure that the potential mutagenic effects of direct exposures to these metabolites could be demonstrated in both mice and rats.

BD exposure levels of 20, 62.5, and 625 ppm were used in this work because these concentrations were carcinogenic in cancer studies conducted in the mouse (Huff et al. 1985; Melnick et al. 1990), whereas the selected exposure concentrations of the racemic mixtures used in the BDO and BDO_2 experiments were based on estimates of the exposure levels that would mimic metabolite blood levels in $B6C3F_1$ mice exposed to 62.5 and 625 ppm BD (that is, blood levels consisting of all stereochemical configurations of BDO and BDO_2 produced in vivo in BD-exposed mice, not just the racemic mixtures). The estimates for the BDO exposure concentrations were based on the physiologically based pharmacologic model of Medinsky and colleagues (1994), which predicted that an exposure of 25 ppm BDO would lead to blood levels for BDO comparable with those achieved by exposure of mice to 625 ppm BD. The exposure concentrations for BDO_2 were based on linear extrapolation from blood levels of BDO_2 that were achieved in earlier exposures of rats and mice to (±)-BDO_2 (Henderson et al. 1996). Mice exposed to 12 ppm BDO_2 for 6 hours had blood levels of approximately 1,000 pmol/g blood. A 4-hour exposure of mice to 62.5 ppm BD resulted in a blood level

of approximately 200 pmol BDO_2/g blood (Thornton-Manning et al. 1995a). The kinetic data in that study indicated that blood BDO_2 concentration was still increasing linearly at the end of a 4-hour exposure. Extrapolating linearly predicts a level of 300 pmol BDO_2/g blood at the end of the 6-hour exposure. Thus, to achieve 300 pmol BDO_2/g blood by direct exposure would require exposures to 300/1,000 × 12 ppm, or 3.6 ppm BDO_2. By linear extrapolation, the equivalent to a 625 ppm BD exposure would be 36 ppm BDO_2. Based on earlier studies in our laboratories, a 6-hour exposure to 36 ppm BDO_2 would be much too high for the survival of animals exposed by inhalation due to toxicity in the nasal passages, tissues in which the highly reactive, water-soluble BDO_2 delivers a high dose. Therefore, we chose to expose the rodents to 4 ppm BDO_2 to represent a rough equivalent to an exposure to 62.5 ppm BD, as well as to 2 ppm BDO_2 as a backup to the higher exposure level.

Rodents were exposed to 0, 62.5, or 625 ppm BD, to 0, 2.5, or 25 ppm BDO, or to 0, 2, or 4 ppm BDO_2 for 4 weeks at 6 hours/day, 5 days/week, using three (or four in the case of BD) multitiered, whole-body exposure chambers (H-2000, Lab Products, Inc., Aberdeen, MD) for exposures to each of the three compounds. The total volume of the H-2000 chamber is 1.7 m^3. Flow rate through each chamber was maintained at 15 ± 2 air changes/hour. All chamber supply air was HEPA-filtered before being introduced into the chamber supply system. Rodents in one chamber received filtered air only as a control group, and rodents in the other chambers received the lower or higher concentration of the test compound. The BD exposures were conducted using BD gas delivered to each chamber from compressed gas cylinders. Flow from each cylinder was controlled via a rotameter. The BDO and BDO_2 vapors were generated with the use of a J-tube generation system. Each exposure was operated for 6 hours plus T_{90} (that is, time to reach 90% of the target concentration) per exposure day. The T_{90} values for each concentration level were determined prior to the start of exposures. Animals were housed within the exposure chambers throughout the experiment, and they had free access to food and water except during the 6-hour exposure periods, when food was removed.

In the J-tube generation system, BDO or BDO_2 was injected into the glass J-tube at a controlled feed rate via a syringe pump. The feed rate was varied to control chamber concentration. The J-tube was heated to 120°C to ensure that the BD-derived epoxy compound (either BDO or BDO_2) was completely vaporized. Nitrogen carrier gas was passed through the J-tube at 2 L/min and was injected into the main dilution chamber.

For each compound, vapor concentration in exposure chambers was monitored by two independent methods. A Foxboro Miran IA infrared spectrometer was used periodically to monitor chamber vapor concentration in real time via infrared absorbance. The Miran is a single-beam, variable-filter infrared spectrometer, and the gas-cell parameters, wavelength, and path length were adjusted for optimal operation for each compound. The second, or backup, quantification of chamber atmosphere differed with test compound. For BD exposures, grab samples were collected three times during each exposure day, and the samples were analyzed by gas chromatography. The BDO atmospheres were similarly quantified by gas chromatography of grab samples. To quantify the BDO_2 atmospheres, bubbler samples were extracted three times during each exposure day from each chamber. Sample trains consisted of two glass impingers connected in series, with each impinger filled with 50 mL of ethyl acetate. Sample trains were on ice and operated at 2 L/min for 1 hour. The bubbler samples were then analyzed by gas chromatography–mass spectrometry.

Chemical-exposed and control mice and rats were killed at specific times after the last exposure by CO_2 asphyxiation followed by exsanguination via cardiac puncture, and the spleen was removed aseptically for isolation of T lymphocytes and measurement of *hprt* MFs. As part of additional collaborative studies, heparinized blood and other target and nontarget tissues for BD-induced carcinogenesis were collected for measurements of BDO- and BDO_2-derived hemoglobin and DNA adducts within the same animals.

Effects of Exposure Duration, Elapsed Time After Exposure, and Exposure Concentration on *hprt* Mutant Frequencies in T Cells from Spleen of Mice and Rats Exposed to BD (Experiments 1–3)

Several experiments were conducted in BD-exposed animals. To assess the accumulation of *hprt* mutations in splenic T cells of mice and rats during repeated exposures to BD, groups of control and exposed animals (n = 6/group) were exposed to 0 or 625 ppm BD for either 2 or 4 weeks, and they were necropsied 4 weeks after the last exposure for isolation of splenic T cells and measurement of MFs (Experiment 1). To characterize differences between mice and rats in changes of *hprt* MFs following BD exposure, groups of animals (n = 4–9/group) were exposed to 0 or 625 ppm BD for 4 weeks and then necropsied at 2, 4, 5, 6, or 8 weeks after exposure for measurement of MFs (Experiment 2). To determine the dose-response relation for *hprt* MFs, groups of mice and rats (n = 6–9/group) were exposed to 0, 20, 62.5, or 625 ppm BD for 4 weeks and then necropsied 4 weeks after exposure for MF measurements (Experiment 3A). For comparison with the MFs obtained in animals exposed to 1,250 ppm BD for 2 weeks as part of Specific Aim 1, groups of mice and rats were removed from exposure at 2 weeks after initial exposures to 0, 20, or 625 ppm BD and necropsied 4 weeks after exposure for MF measurements in splenic T cells (Experiment 3B).

Effects of Elapsed Time After Exposure and Exposure Concentration on *hprt* Mutant Frequencies in T Cells from Spleen of Mice and Rats Exposed to BDO (Experiments 4 and 5)

Parallel studies were conducted in mice and rats to define the change in *hprt* MFs over the course of exposures to BDO (Experiment 4) and to ascertain the dose-response relation for *hprt* MFs (Experiment 5). In these experiments, animals were exposed concurrently to 0, 2.5, or 25 ppm BDO for 4 weeks and necropsied at 1, 3, 5, 7, or 9 weeks after the last exposure for mice (n = 3–12/group), or at 1, 3, 5, or 7 weeks after the last exposure for rats (n = 3–12/group).

Effects of Elapsed Time After Exposure and Exposure Concentration on *hprt* Mutant Frequencies in T Cells from Spleen of Mice and Rats Exposed to BDO_2 (Experiments 6 and 7)

Parallel studies were conducted in mice and rats to describe the change in *hprt* MFs over the course of exposures to BDO_2 (Experiment 6) and to determine the dose-response relation for *hprt* MFs (Experiment 7). In these experiments, animals were exposed to 0, 2, or 4 ppm BDO_2 for 4 weeks and necropsied at 1, 2, 4, 6, 7, or 9 weeks after the last exposure for isolation of splenic T cells and measurement of MFs (n = 4–6/group). The control animals in these collective studies with BD, BDO, and BDO_2 (Experiments 1–7) included mice and rats necropsied before initial exposure and groups of animals exposed to chamber supply air and then necropsied at the same time points after the last exposure as the analogous exposed groups.

Isolation and Culture of *hprt* Mutant T Cells

The general procedures for isolating T lymphocytes from spleen and culturing *hprt* mutant T cell clonal colonies were the same as described for Specific Aim 1 with one exception. For overnight stimulation and subsequent outgrowth of mouse T cells, both priming medium and cloning medium were modified by adding 10% rat T-STIM™, a conditioned medium derived from concanavalin A–stimulated rat splenocyte cultures. Supplemented medium for priming and growing the rat T cells was the same as that used in Specific Aim 1, and it did not contain rat T-STIM™. The *hprt* mutant clonal colonies from spleen of both control and exposed mice were collected and frozen for molecular analysis.

SPECIFIC AIM 3

Molecular Analysis of Mouse and Rat T Cell Clones for Mutations in *hprt* Exon 3 (Experiments 1–5)

Mutant T cell clones collected from control mice, mice and rats exposed to BD, and mice and rats exposed to BDO_2 were evaluated for mutations in the exon 3 region of *hprt*. Mutational spectra data could not be generated for BDO-exposed animals because too few *hprt* mutants were recovered from BDO-exposed mice and because no significant mutagenic response was noted in BDO-exposed rats (in Results section, see Experiments 4 and 5 of Specific Aim 2).

The procedures used to identify and characterize mutations in the exon 3 region of mouse and rat *hprt* were performed as previously described (Skopek et al. 1992; Walker and Skopek 1993a; Mittelstaedt et al. 1995) but with minor modifications. In summary, genomic DNA from each mutant clone was extracted using a Tween 20–proteinase K digestion mix, aliquots of DNA were used to PCR-amplify *hprt* exon 3, mutations in the low-temperature and high-temperature melting domains of exon 3 were identified and purified using DGGE, and finally, DNA eluted from heteroduplex bands excised from DGGE gels was PCR-amplified and sequenced.

All of the PCR primers previously developed for the amplification of mouse and rat *hprt* exon 3 (Skopek et al. 1992; Walker and Skopek 1993a; Mittelstaedt et al. 1995) were used in this study, except for the 3′ primers used by Mittelstaedt and colleagues (1995) for the first-round PCR of an extended exon 3 fragment in the rat (primer R308R) and for the second-round PCR to analyze the low-temperature melting domain of rat exon 3 (primer H3). The 3′ primer (5′-TAATTATACTTACACAGTA-3′) used in lieu of R308R and H3 was complementary with the last five bases of exon 3 and the first 14 bases of intron 3 on the coding strand. DNA sequences for the other rat-specific PCR primers used in the current study were kindly provided by Mittelstaedt and colleagues (1995) prior to publication of this information.

Exon 3 PCR products from both mice and rats were analyzed for mutation by DGGE as previously described for the mouse (Skopek et al. 1992), with the exception that the low-temperature melting domain products were screened using a 27% to 36% denaturing gradient. Instead of extracting DNA (Skopek et al. 1992) or precipitating DNA (Mittelstaedt et al. 1995) from PCR reactions to be analyzed by DGGE (both primary gels for mutant identification, and secondary gels for purification of mutant–wild-type heteroduplexes), EDTA (5 µL of a 50 µM solution) was added to the PCR products (23 µL of a 30-µL PCR reaction) to inactivate *Taq* polymerase prior to heteroduplex formation. Heteroduplexes that overlapped on primary DGGE gels were subsequently resolved into upper and lower bands using narrower denaturing gradients for both the low-temperature and high-temperature domains (that is, 31% to 36% and 44% to 53%, respectively). Each heteroduplex on a primary DGGE gel was resolved into two

mutant–wild-type heteroduplexes on a secondary purification DGGE gel, and each set of four bands on a purification DGGE was excised and eluted to provide a template for strand-biased PCR to identify a single mutation (Skopek et al. 1992). All four strand-biased PCR reactions were run through Centricon 50 concentrators (Amicon, Inc., Beverly, MA) to filter out the primers, and the filtrates were subsequently vacuum-dried and resuspended in 20 µL sterile water as templates for single-strand PCR reactions that used the appropriate 5′ primer. Single-strand PCR products were filtered through CentriCep spin columns (Princeton Separations, Adelphia, NJ) and aliquots of these PCR products were then sequenced using the sequenase-dideoxy protocol specified by PE Applied Biosystems (Foster City, CA). This approach produced a sequence for each of the four heteroduplexes obtained from purification DGGE gels (that is, two identical sets of heteroduplexes for the same mutation), with two of the sequences (corresponding to a pair of either upper or lower heteroduplex bands) usually having a wild-type sequence and the other two sequences (corresponding to the opposite pair of heteroduplex bands) having the same mutation in exon 3.

SPECIFIC AIM 4

Propagation of *hprt* Mutant T Cell Clones from Spleen of Mice

6-Thioguanine–resistant T cell clones from control and exposed mice necropsied 6 weeks after a 4-week exposure to 625 ppm BD (exposure of 6 hours/day, 5 days/week; see Specific Aim 2, Experiment 2) were used in an experiment evaluating the effect of concanavalin A stimulation on T cell colony expansion. Of the *hprt* mutant colonies, 48 colonies from control and 72 colonies from BD-exposed mice were propagated sufficiently for molecular analysis by reverse transcriptase (RT)–PCR of mRNA or exon-specific PCR of genomic DNA for mouse *hprt*. Details of the propagation procedure are presented elsewhere (Meng et al. 1998b).

Molecular Analysis of Expanded T Cell Clones for Mutations in the *hprt* Gene of Mice

Propagated mutant T cell clones from control and BD-exposed mice were evaluated for mutations in *hprt* cDNA of the mouse gene using an RT-PCR procedure modified from an unpublished method developed for mouse *hprt* by Dr. Tao Chen (protocol kindly provided by R. H. Heflich, National Center for Toxicological Research). Mutant clones that produced *hprt* cDNA were further analyzed by DNA sequencing, whereas clones that did not yield *hprt* cDNA were then analyzed for the presence of mRNA by RT-PCR of the β-*actin* gene.

For preparation of total RNA, frozen pellets of expanded clones were thawed on ice and $1–10 \times 10^3$ cells were transferred to 500-µL Eppendorf tubes containing 10 µL RNase-free H_2O (Promega, Madison, WI), 0.4% RNasin (Promega), and 2.5% Nonidet P-40 (Sigma, St. Louis, MO). The cells were mixed with a pipette tip to assist in cell lysis and incubated for 20 minutes on ice. The cell lysate was then used as the source of total RNA for RT-PCR reactions.

For the initial RT-PCR amplification of *hprt*, 4 µL of cell lysate was used in a final volume of 20 µL RT-PCR solution, including 2 µL of 10X VM buffer (10 mM $MgCl_2$), 0.4 µL of dNTP (25 mM) (Promega), 0.4 µL of Oligo dT (0.5 µg/µL) (PE Applied Biosystems), 1.0 µL of *hprt*-specific 5′ primer (10 µM; M101F: 5′-TTA CCT CAC TGC TTT CCG GA-3′) and 3′ primer (10 µM; M901R: 5′-GAT GGC CAC AGG ACT AGA AC-3′), 0.4 µL of AMV reverse transcriptase (5 U/µL) (Promega), 0.4 µL of *Tfl* DNA polymerase (5 U/µL) (Promega), and 10.4 µL of sterile double-deionized H_2O. The reaction mixture was overlaid with mineral oil and incubated in a Perkin Elmer 480 thermocycler for 45 minutes at 48°C and then for 2 minutes at 94°C, followed by 40 cycles of 30-second denaturation at 94°C, 1-minute annealing at 55°C, and 2-minute extension at 68°C, with the last cycle containing a 7-minute extension at 68°C. The product from this reaction was diluted 1:100 in sterile H_2O and 1 µL of this dilution was used as a cDNA template in a nested PCR. The 30 µL of nested PCR reaction contained 3 µL of 10X VM buffer (27.5 mM $MgCl_2$), 1 µL of *hprt*-specific 5′ primer (10 µM; M102F: 5′-GGC TTC CTC CTC AGA CCG CT-3′) and 3′ primer (10 µM; M902R: 5′-GGC AAC ATC AAC AGG ACT CC-3′), 0.3 µL of AmpliTaq DNA polymerase (5 U/µL) (Boehringer Mannheim, Indianapolis, IN), and 22.7 µL of sterile ddH_2O. The reaction mixture was overlaid with mineral oil and the following PCR cycle program was applied: 4 minutes at 94°C; 30 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes; and the last cycle contained a 7-minute extension at 72°C. An aliquot of 5 µL of the nested PCR reaction volume was analyzed on an 8% polyacrylamide gel.

For direct sequencing of *hprt* PCR products, the remaining nested PCR reactions were run through Centricon 50 concentrators (Amicon, Inc., Beverly, MA); the filtrates were vacuum-dried and resuspended in 20 µL sterile water as templates for single-strand PCR reactions run in both directions with the use of the appropriate 5′ primer (M102F) or 3′ primer (M902R). Single-strand PCR products were filtered through CentriCep spin columns, and aliquots of these PCR products were then sequenced using the sequenase-dideoxy protocol specified by PE Applied Biosystems.

STATISTICAL ANALYSES

Although the literature on analyses associated with potency experiments is extensive, the bulk of it is confined to considering parallel line assays where responses are observed at differing concentrations. The current investigation, on the other hand, involves an assay where nonlinear responses are taken at a single concentration but are observed over multiple time points. A literature search failed to identify an extant technique for estimating the mutagenic potency in temporal data of this type. We therefore adopted the technique of summarizing the response in terms of total mutagenicity by fitting and then integrating a polynomial model with respect to time. This objective was accomplished by the following steps.

Fitting a Nonlinear Model (Step 1)

A robust regression (Paulson and Delehanty 1982) was used to estimate the parameters of a cubic polynomial on the log of the MF data. The log transformation was selected in order to make the residuals as Gaussian as possible in distribution and to correct for heterogeneity in variance. The degree of the polynomial was selected by considering the significance of *F* tests for coefficients of the treatment group within a higher-order model orthogonalized with respect to time. The selected order was retained in the control group. Robust regression was employed to minimize the influence of outliers in the data by down-weighting observations with large residuals. The effectiveness of applying a robust regression in minimizing outlier effect in MF values was tested using the MF data set for splenic T cells from BD-treated mice (see Results section, Specific Aim 1).

Integration (Step 2)

The areas under the fitted curves were found by transforming each modeled polynomial back to the original scale (exponentiating) and, because the integrand had no closed form, integrating each curve using numerical quadrature (Press et al. 1992). The trapezoidal rule was used as the integration technique. The mutagenic potency was represented by the difference in the areas under the curves for exposed and for control animals.

Mutagenic Potency Confidence Intervals (Step 3)

Confidence intervals (CIs) for the Step 1–modeled mutagenic potencies, which are also unavailable in closed form, were obtained from Monte Carlo simulations. Simulated MF data were created by adding normal pseudorandom variates with zero mean and variance equal to that observed in the experimental data. Cubic polynomials and the areas under them, and hence the mutagenic potencies, were then found for this simulated data as in Step 2. The CIs were formed by selecting the 2.5 and 97.5 percentiles as endpoints.

Hypothesis Testing

The *p* values used to describe the differences in MF values between groups of control and exposed animals at specific time points in various experiments with BD (Specific Aims 1 and 2), BDO (Specific Aim 2), and BDO_2 (Specific Aim 2) were formed by the Mann-Whitney *U* statistic (Hollander and Wolfe 1973). To compare frequencies of different classes of mutations identified by RT-PCR amplification of *hprt* in mutant clones from control and BD-exposed mice, the chi-square test was used to evaluate expected percentages under the null hypothesis—that percentages should occur in the same proportions in both groups (Hollander and Wolfe 1973). A Fisher exact test for homogeneity of proportions of mutation types in control mice versus BD- or BDO_2-exposed mice was performed. Also, a Fisher exact hypothesis test was used to determine if individual types of base substitutions were significantly different between control and treatment (BD or BDO_2) groups; the computations were performed under the null hypothesis that percentages should occur in the same proportions in treatment and control groups. The latter test was performed in the fashion of Carr and Gorelick (1996), except that MF was not corrected to "mutation frequency" for each mutation type for reasons related to T cell kinetics in the mouse (see Discussion and Conclusions section). A *p* value less than 0.05 was considered significant.

RESULTS

SPECIFIC AIM 1

Effects of BD Exposure on Toxicity and T Cell Cloning Efficiency in Mice and Rats

Two weeks of exposure to 1,250 ppm BD (6 hours/day, 5 days/week) caused no overt signs of toxicity in exposed animals; the average inhalation chamber concentration for the entire exposure period was 1,248.9 ± 9.8 ppm BD for mice and 1,257.2 ± 16.2 ppm BD for rats. The cloning efficiencies of T cells isolated from thymus and spleen of BD-exposed mice and rats were indistinguishable from control values, which came both from animals killed before exposure and animals killed after sham exposure. Cloning efficiencies in thymus of control and BD-exposed mice (0.5–2.1%; $n = 108$) and rats (0.5–1.5%; $n = 110$) were similar. However, cloning efficiencies in spleen of control and BD-exposed mice (2.1–9.4%; $n = 108$) were lower than

both sets of values for rats (3.5–29.1%; n = 102). The mean [± SD] *hprt* MFs in T cells taken from control mice were 2.4 [± 0.5] × 10^{-6} (n = 26) mutants per clonable cell in thymus and 1.9 [± 0.5] × 10^{-6} (n = 34) in clonable spleen cells. Similar MFs were measured in thymus (2.1 [± 0.3] × 10^{-6}; n = 24) and spleen (2.2 [± 0.8] × 10^{-6}; n = 43) from control rats. These cloning efficiency and MF values resemble those reported previously for lymphocytes from thymus and spleen of both untreated B6C3F$_1$ mice (Skopek et al. 1992; Walker and Skopek 1993a; Walker et al. 1997, 1999) and untreated F344 rats (Aidoo et al. 1991, 1994).

Effects of Exposure Duration on *hprt* Mutant Frequencies in T Cells from Thymus of Mice and Rats Exposed to BD (Experiment 1)

The induction and accumulation of *hprt* mutations in thymic T cells were assessed in mice and rats necropsied 2 weeks after a 1-week or 2-week exposure to 1,250 ppm BD (Figure 1). Significantly elevated MFs were apparent in exposed mice following 1 week of exposure, and MFs continued to increase through the end of the second week of exposure. The respective MF values in exposed mice were 3-fold (p = 0.008) and 5-fold (p = 0.001) higher than values in controls. The mutagenic effects of BD were not as marked in cells from rat thymus. No increase over background MF was observed after 1 week of exposure and only a 1.7-fold increase (p < 0.001) over control values was observed after 2 weeks of exposure.

Effects of Elapsed Time After Exposure on *hprt* Mutant Frequencies in T Cells from Thymus and Spleen of Mice and Rats Exposed to BD (Experiment 2)

The effect of elapsed time after BD exposure on *hprt* MFs in thymic T cells was evaluated following a 2-week exposure of mice and rats to 1,250 ppm BD. In mice, mean [± SE] MFs were significantly increased at 1 week after exposure (p = 0.008), reached maximum levels at 2 weeks after exposure (11.3 [± 2.4] × 10^{-6}; p = 0.001), and then declined toward background level through 10 weeks after the last exposure (Figure 2). In rats, MFs were significantly increased at 2 weeks after the last exposure (p < 0.001), achieved peak levels at 3 weeks after exposure (4.9 [± 1.2] × 10^{-6}; p = 0.036), and then decreased gradually to background level by 10 weeks after exposure (Figure 2). The maximum average MFs in mouse and rat thymus were 5- and 2.5-fold greater than their respective control, or background, values. A similar pattern of mutant expression has been observed in thymic T cells from male B6C3F$_1$ mice of similar age following in vivo exposures to ethylnitrosourea (Walker and Skopek 1993b).

The phenotypic expression of *hprt* MFs was also determined in T cells isolated from spleen of the same BD-exposed animals. In mice, mean [± SE] *hprt* MFs were significantly increased at 2 weeks after the last exposure (p < 0.001), reached a peak at 5 weeks after exposure (19.7 [± 1.9] × 10^{-6}; p = 0.003), and then decreased over the next 3 weeks to lower, but still elevated, levels (p = 0.021) (Figure 3). In rats, mean [± SE] *hprt* MFs were significantly increased at 2 weeks after

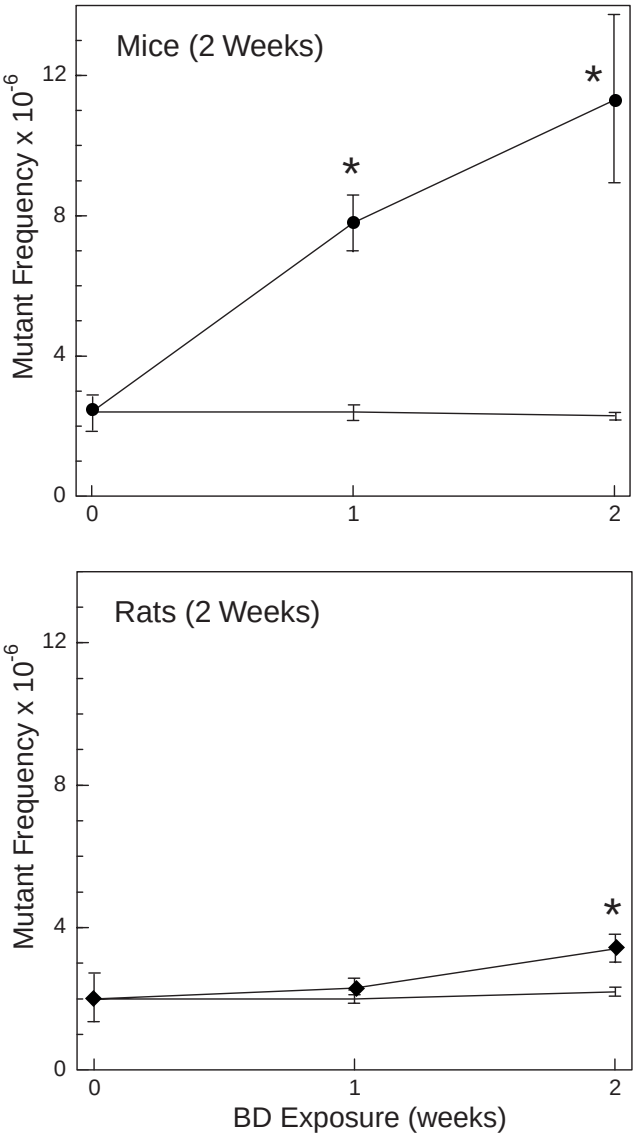

Figure 1. Relation between exposure duration and *hprt* MF in thymic T cells from female B6C3F$_1$ mice and F344 rats exposed to 0 or 1,250 ppm BD for 1 or 2 weeks. Groups of animals were necropsied just prior to the first day of exposure or two weeks after cessation of a 1- or 2-week exposure. Note the significant increase in MF in mice through the 2 weeks of exposure and in rats after 2 weeks of exposure. Exposed mice (●) and rats (◆); control animals (no markers); data points are means with error bars (n = 4–8 control or 10 exposed animals). An asterisk (*) indicates a significant change compared with control values.

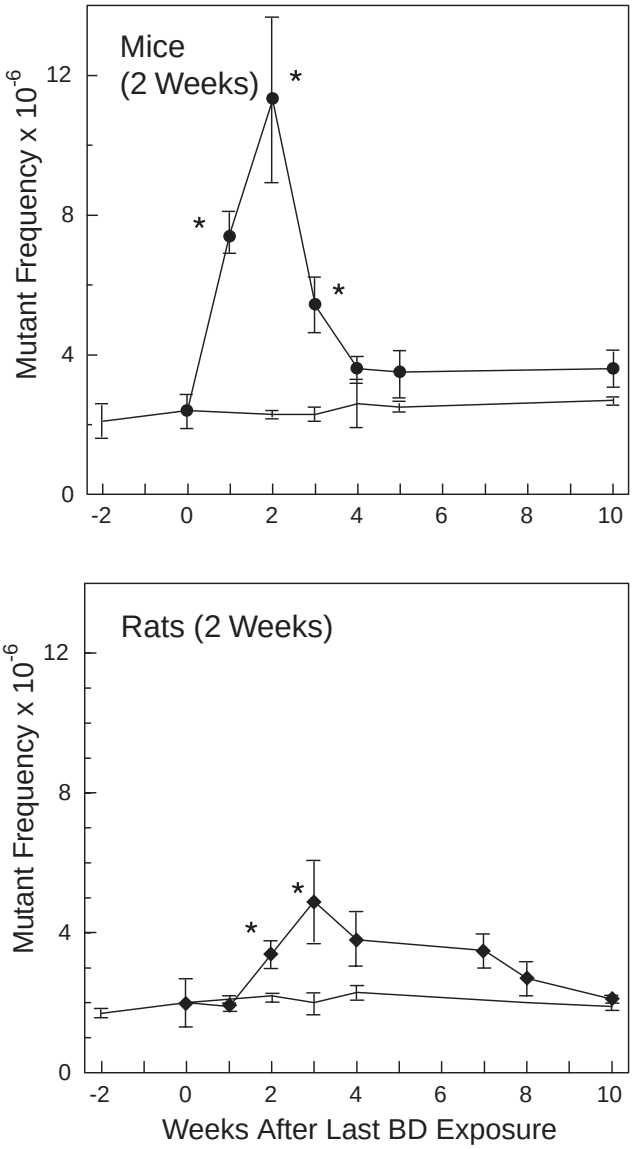

Figure 2. Relation between elapsed time after last BD exposure and *hprt* MF in thymic T cells from female B6C3F$_1$ mice and F344 rats exposed to 0 or 1,250 ppm BD for 2 weeks. Groups of animals were necropsied weekly or biweekly up to 10 weeks after last exposure. Note the significant increase in MF in mice during the first, second, and third weeks after exposure, and in rats during the second and third weeks after exposure. Exposed mice (●) and rats (◆); control animals (no markers); data points are means with error bars (n = 4–8 control or 10 exposed animals). An asterisk (∗) indicates a significant change compared with control values.

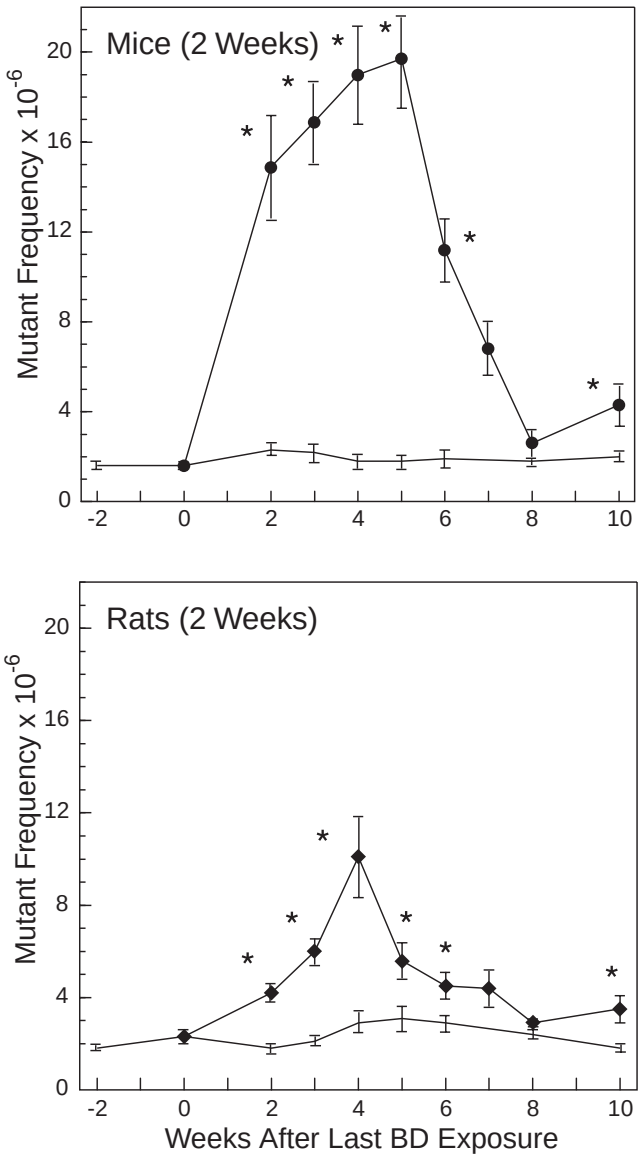

Figure 3. Relation between elapsed time since last BD exposure and *hprt* MF in splenic T cells from female B6C3F$_1$ mice and F344 rats exposed to 0 or 1,250 ppm BD for 2 weeks. Groups of animals were necropsied weekly or biweekly up to 10 weeks after last exposure. Note the significant increase in MF in mice and rats 2 through 6 weeks after exposure and at 10 weeks after exposure. Exposed mice (●) and rats (◆); control animals (no markers); data points are means with error bars (n = 4–8 control and 10 exposed animals). An asterisk (∗) indicates a significant change compared with control values.

the last exposure ($p < 0.001$), achieved peak values at 4 weeks after exposure (10.1 [± 1.8] × 10^{-6}; $p = 0.005$), and then gradually declined to lower, but still elevated, levels ($p = 0.005$). A similar pattern of mutant manifestation has been found in T cells from spleen of conventional and *lacI* transgenic B6C3F$_1$ mice of similar age following exposures to ethylnitrosourea or cyclophosphamide (Walker and Skopek 1993b; Walker et al. 1999). However, the phenotypic expression of *hprt* mutant T cells in rat spleen has not been previously investigated in rats treated with a mutagen at 4 to 5 weeks of age.

Although F344 rats are an inbred strain and B6C3F$_1$ mice are a hybrid of two inbred strains, considerable variation was observed in MF among animals exposed to BD. The average coefficient of variation (CV) of MFs in control animals was 0.24, indicating a relatively small interindividual variation in the absence of exposure. In BD-exposed mice and rats, the CV

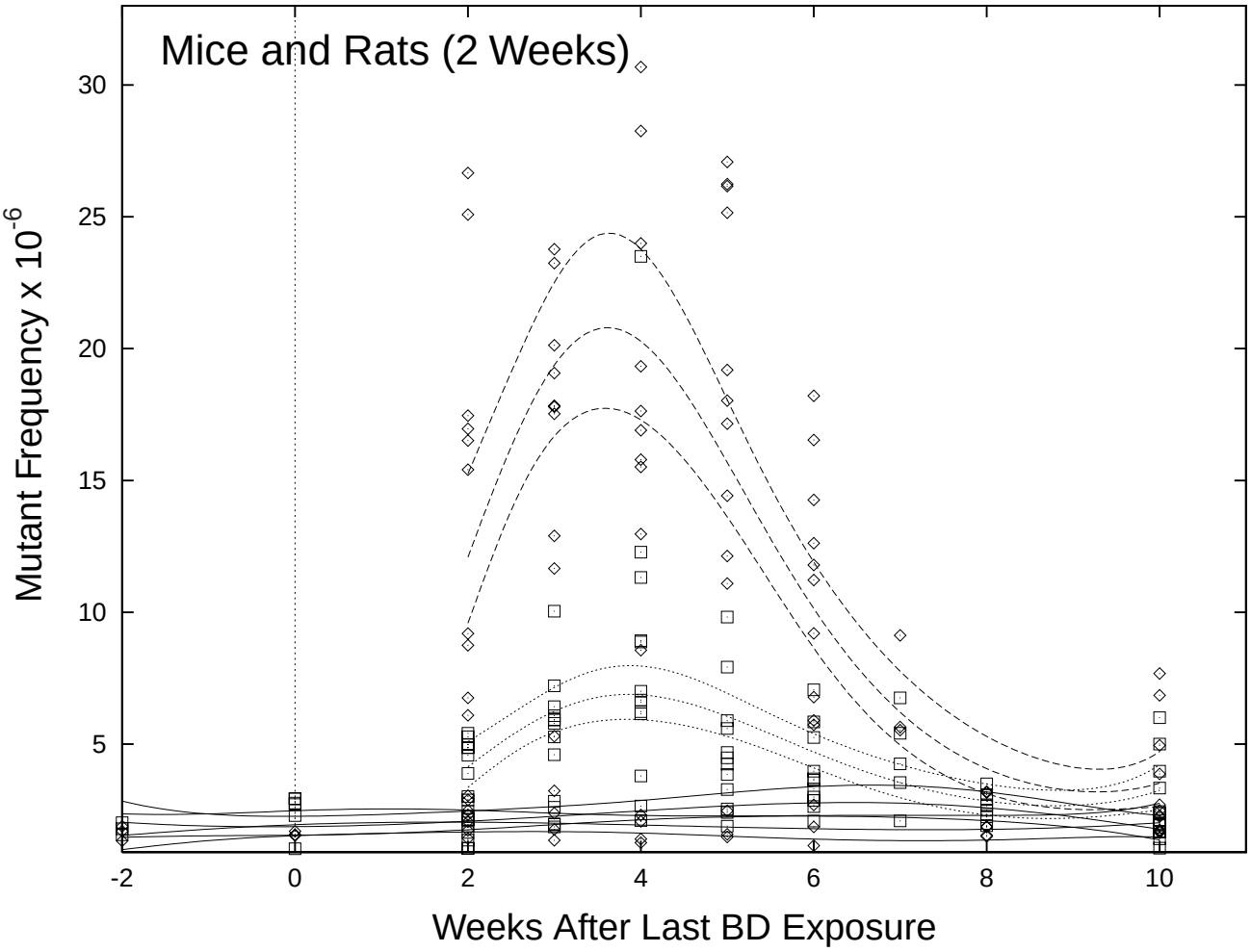

Figure 4. Curves of individual *hprt* MF data obtained for splenic T cells following exposure of female B6C3F$_1$ mice and F344 rats to 0 or 1,250 ppm BD for 2 weeks. Groups of animals were necropsied weekly or biweekly up to 10 weeks after exposure. Data obtained from necropsied mice and rats were used to derive robustly estimated polynomial models and 95% CIs computed from the associated *t* distribution. Areas under the estimated curves were used to compute the mutagenic potency for each species (see Methods and Study Design section). For exposed mice: curve and CIs ----; data ◇. For exposed rats: curve and CIs ·····; data ⊡. For control animals: curve and CIs —.

for MFs at different time points after the last exposure ranged narrowly around 0.39 when significant outliers were excluded. The interindividual variation in *hprt* MFs observed in the present experiments (namely, CV of 0.33) resembled values found previously in BD-exposed (625 ppm BD for 2 weeks, 6 hours/day, 5 days/week) preweanling mice (Cochrane and Skopek 1994b). Potential reasons for this variation among mutagen-treated animals have been discussed elsewhere (Cochrane and Skopek 1994b).

Mutagenic Potency of BD Exposure in T Cells from Spleen of Mice and Rats

Curves were fit to individual MF data for splenic T cells (Figure 4), with the difference in the area under the curves for treated compared with control mice or rats representing the mutagenic potency of BD in that species. Table 1 presents the estimated mutagenic potencies and Monte Carlo CIs in mice compared with rats. These values indicate that the mutagenic potency of BD is significantly greater (4.4-fold) in splenic T cells of female B6C3F$_1$ mice (69.6) than it is in female F344 rats (15.9) when curves were derived from MFs observed between 2 and 8 weeks after exposure.

An alternative approach for estimating species difference in mutagenic potency is to determine the ratio of the induced MFs in mice compared with rats for each of the time points between 2 and 8 weeks after exposure (that is, ratios in splenic T cells of 5.3, 3.8, 2.4, 7.2, 6.0, and 2.0, at 2, 3, 4, 5, 6, and 8 weeks after exposure, respectively), and then to obtain an average ratio representing the species difference in mutagenic response over time. In this case, the sum of the ratios divided by the six time points in the

Table 1. Estimated Mutagenic Potencies and Monte Carlo CIs[a] Based on *hprt* Mutant Frequencies in Splenic T Cells of Control and Exposed Female B6C3F$_1$ Mice and F344 Rats After Exposure to 1,250 ppm BD[b]

Species	Mutagenic Potency[c]	95% CI[d]
Mouse	69.6	61.1, 78.7
Rat	15.9	11.9, 19.8

[a] The computations for estimating mutagenic potency and CIs are described in the Methods and Study Design section. Mutant frequencies measured in animals necropsied between 2 and 8 weeks after exposure were used in the computations for this table (see Figure 4).

[b] Exposure was for 6 hours/day, 5 days/week, for 2 weeks.

[c] Mutagenic potency is a relative value that represents the number of mutant cells passing through the spleen over the period of time being evaluated; that is, mutagenic potency approximates the [number of *hprt* mutants/number of wild-type cells] × [number of wild-type cells passing through the spleen/unit of time].

[d] The 95% CIs for the mouse and rat mutant frequency data do not overlap, which demonstrates a significant increase in the mutagenic potency in exposed mice compared with exposed rats.

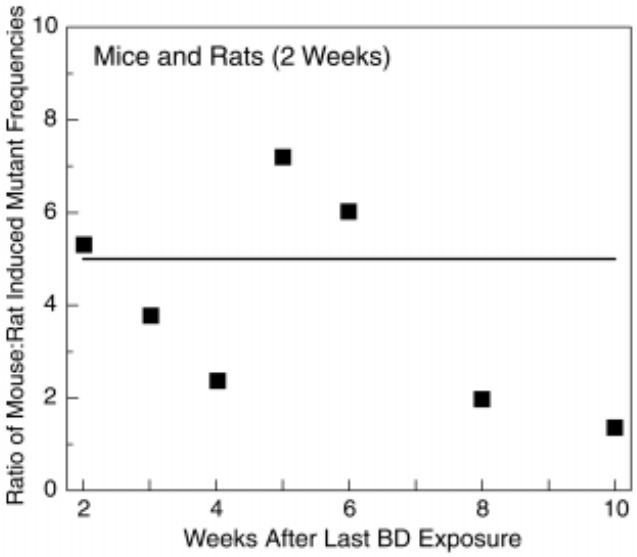

Figure 5. Comparison of the mutagenic response in splenic T cells from female B6C3F$_1$ mice and F344 rats following 2 weeks of exposure to 1,250 ppm BD. The horizontal line represents the ratio of the mutagenic potency of BD (under these exposure conditions) in mice compared with rats necropsied between 2 and 10 weeks after exposure. The individual points (■) are ratios of induced *hprt* MFs in mice compared with rats at different time points after exposure. Comparing the mutagenic response to BD using the ratio of MFs at a single time point will either over- or underestimate the difference of mutagenic potency in exposed mice compared with rats.

phenotypic expression curve (that is, 26.7/6) indicates that the mutagenic response in BD-treated mice was 4.5-fold greater than that observed in rats. However, the curve in Figure 5 shows that comparing the average of induced MFs in mice with that for rats at any single postexposure time point would either overestimate or underestimate the species difference in the mutagenic potency of BD.

SPECIFIC AIM 2

Exposure of Mice and Rats to BD, BDO, and BDO_2

The actual exposure concentrations for BD, BDO, and BDO_2 were determined as described in the Methods and Study Design section using periodic samplings of inhalation chamber atmospheres. In the case of BDO, a preliminary 6-hour exposure of female mice to 25 ppm BDO was conducted to determine if the blood levels achieved were similar to those in mice exposed to 625 ppm BD for the same exposure period. The blood levels of BDO in three mice exposed for 6 hours to 25 ppm BDO were 3.70 ± 0.06 nmol/g (mean ± SD). This mean level compared favorably with previously published values indicating that mice exposed to 625 ppm BD for 6 hours would have blood levels of 3.7 nmol BDO/g blood (Himmelstein et al. 1994) or 3.3 nmol BDO/g blood (Thornton-Manning et al. 1995a). In the 4-week exposure studies, the mean ± SD inhalation concentrations for the exposure periods were 20.06 ± 1.04 ppm for 20 ppm BD, 62.73 ± 2.31 ppm for 62.5 ppm BD, and 623.27 ± 50.86 ppm for 625 ppm BD (n = 120 measurements/dose); 2.65 ± 0.74 ppm for 2.5 ppm BDO, and 29.95 ± 1.80 ppm for 25 ppm BDO (n = 120 measurements/dose); and 2.04 ± 0.52 ppm for 2 ppm BDO_2, and 3.84 ± 0.65 ppm for 4 ppm BDO_2 (n = 60 measurements/dose).

Cloning Efficiency of T Cells from Spleen of Control and Exposed Mice and Rats

Table 2 presents cloning efficiency data for control and exposed mice in the *hprt* mutant T cell manifestation and dose-response studies with BD, BDO, and BDO_2. Cloning efficiencies in control mice were fairly consistent among these studies except in the mutant T cell manifestation study of BDO-exposed mice. Cloning efficiency values in both control and BDO-exposed mice were generally lower than cloning efficiency values obtained in other experiments with BD, BDO, and BDO_2, suggesting that this deviation was related to variations among lot numbers of culture supplements (see Results section, Specific Aim 4). The average cloning efficiency in control mice from the combined studies of BD, BDO, and BDO_2 was 16.4% (n = 93).

The average cloning efficiencies in control and exposed mice were similar in the experiments with BD and BDO; however, the average cloning efficiencies in BDO_2-treated mice were significantly lower than those observed in control mice (p = 0.016; Table 2). The lower cloning efficiencies in

Table 2. Cloning Efficiencies of Splenic T Cells in Control and BD-, BDO-, or BDO_2-Exposed Mice from *hprt* Mutant T Cell Manifestation and Dose-Response Experiments[a]

	Cloning Efficiency (%)			
	Control Mice		Exposed Mice	
Exposure	Mean	Range	Mean	Range
Mutant Manifestation Experiments				
BD (0 or 625 ppm)	16.3	10.9–28.3	15.2	6.0–24.1
BDO (0 or 25 ppm)	12.8	9.3–25.5	11.2	7.8–15.0
BDO_2 (0 or 2 ppm)	16.9	9.9–24.1	13.6[b]	7.3–18.4
BDO_2 (0 or 4 ppm)	16.9	9.9–24.1	12.8[b]	9.3–18.7
Dose-Response Experiments				
BD (0, 20, 62.5, 625, or 1,250 ppm)	17.9	12.4–28.3	16.9	11.9–22.4
BDO (0, 2.5, or 25 ppm)	18.1	9.6–25.5	16.5	6.7–28.3
BDO_2 (0, 2, or 4 ppm)	18.7	9.9–24.1	12.3[b]	6.4–18.4

[a] Female $B6C3F_1$ mice (4–5 weeks old) were exposed to BD, BDO, or BDO_2 for 4 weeks by inhalation (6 hours/day, 5 days/week). Groups of control and exposed mice were necropsied at 2, 4, 5, 6, or 8 weeks after BD exposure; 1, 3, 5, 7, or 9 weeks after BDO exposure; or 1, 2, 4, 6, 7, or 9 weeks after BDO_2 exposure. T cells from spleen of mice were isolated as described in the Methods and Study Design section, primed with concanavalin A, and cultured in medium supplemented with rat T-STIM™ and IL-2 in the presence of 4×10^4 feeder cells/well in 96-well microtiter plates. Cloning efficiency plates were evaluated for cell growth at 8–10 days after plating, and the cloning efficiency values were calculated as previously described (Skopek et al. 1992).

[b] Significant difference from control mice ($p = 0.016$ for each significant comparison) by the Mann-Whitney *U* stastistic.

BDO_2-exposed mice were probably due to increased cytotoxicity of BDO_2 in mouse T cells, as reflected by significantly decreased total lymphocyte counts in peripheral blood (that is, a decrease of 60% at 4 ppm BDO_2) (Henderson et al. 1997) and reduced recovery of lymphocytes from spleen of mice necropsied immediately after cessation of BDO_2 exposure.

The average cloning efficiency in control rats was 30.0% ($n = 87$) in studies with BD, BDO, and BDO_2, and no significant differences were noted in the cloning efficiencies obtained in controls compared with those of exposed rats. These cloning efficiency values are higher than those reported previously for lymphocytes from spleen of F344 rats (Aidoo et al. 1991, 1993; Meng et al. 1998a; see Results section, Specific Aim 1).

hprt Mutant Frequencies in T Cells from Spleen of Control Mice and Rats

Average *hprt* MFs were determined in splenic T cells isolated from control mice and rats (preexposure and sham-exposed animals) in studies of BD, BDO, and BDO_2. (Individual MF values for each control mouse and rat and for various treated animals are shown later in Figures 13 through 17.) In experiments with BD, the mean [± SD] *hprt* MFs in control animals were 2.1 [± 0.6] × 10^{-6} ($n = 40$) in mice and 2.8 [± 0.8] × 10^{-6} ($n = 36$) in rats. The sham-exposed mice and rats for BDO exposures had average MFs of 1.8 [± 0.6] × 10^{-6} ($n = 26$) and 2.6 [± 0.7] × 10^{-6} ($n = 24$), respectively. Average MFs in controls for BDO_2 exposures were 1.8 [± 0.5] × 10^{-6} ($n = 27$) for mice and 2.4 [± 0.6]×10^{-6} ($n = 27$) for rats. In these studies, the average background *hprt* MFs in mice and rats resembled those reported previously for T cells from spleen of untreated mice (Skopek et al. 1992; Cochrane and Skopek 1994b; Walker et al. 1996, 1997) and rats (Aidoo et al. 1991, 1993; Meng et al. 1998a) (see Results section, Specific Aim 1).

Effects of Exposure Duration, Elapsed Time After Exposure, and Exposure Concentration on *hprt* Mutant Frequencies in T Cells from Spleen of Mice and Rats Exposed to BD (Experiments 1–3)

The effects of duration of exposure to BD on the accumulation of *hprt* mutations in splenic T cells were assessed by comparing the MFs in animals necropsied 4 weeks after exposure to either 0 or 625 ppm BD for durations of 2 or 4 weeks (Experiment 1; Figure 6). Significantly elevated MFs were apparent in BD-exposed mice following 2 weeks of exposure, and MFs continued to increase through 4 weeks of exposure, with respective MF values 5.0-fold ($p < 0.001$) and 10.4-fold ($p < 0.001$) higher than those in control mice. The mutagenic effects of BD were not as marked in rat splenic T cells. No increase over background MFs was observed after 2 weeks of exposure,

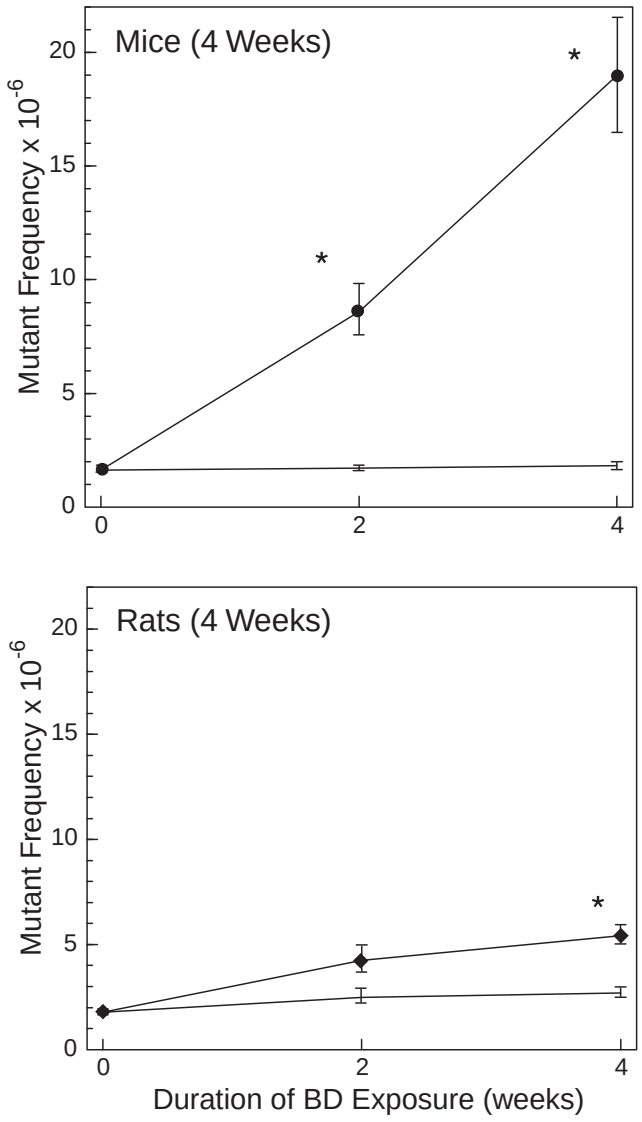

Figure 6. Relation between exposure duration and *hprt* MF in splenic T cells from female B6C3F$_1$ mice and F344 rats exposed to 0 or 625 ppm BD for 2 or 4 weeks. Groups of animals were necropsied prior to the first day of exposure or four weeks after the end of 2 or 4 weeks of exposure. Note the significant increase in MF in mice throughout the 4 weeks of exposure and in rats at 4 weeks of exposure. Exposed mice (●) and rats (◆); control animals (no markers); data points are means with error bars (n = 6 per group). An asterisk (∗) indicates a significant change compared with control values.

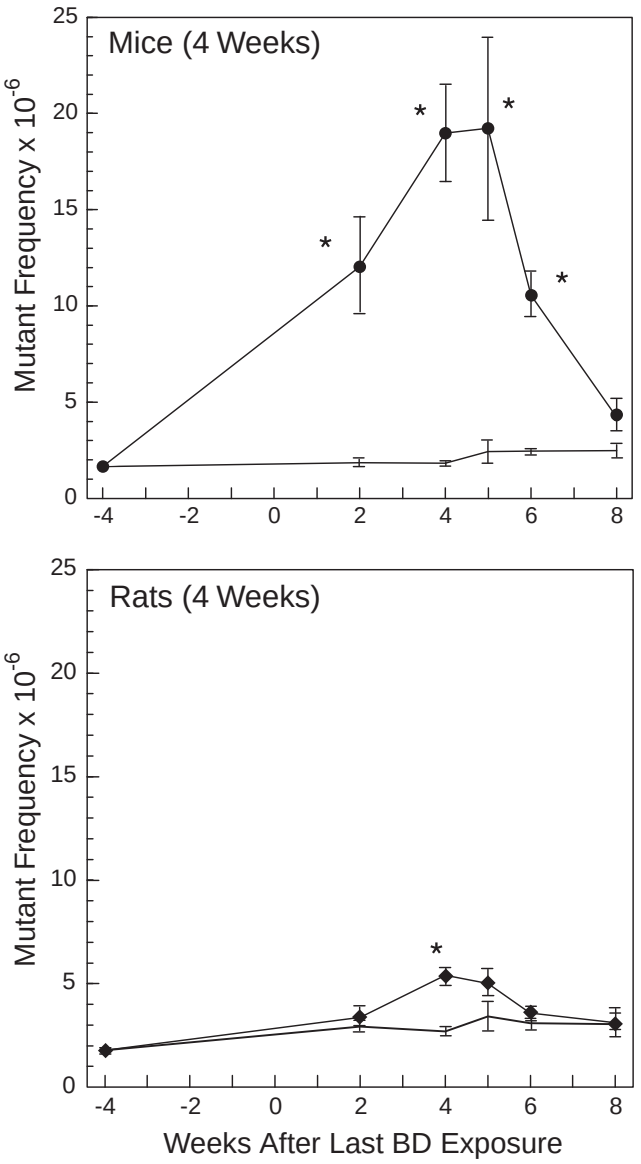

Figure 7. Relation between elapsed time after last BD exposure and *hprt* MF in splenic T cells from female B6C3F$_1$ mice and F344 rats exposed to 0 or 625 ppm BD for 4 weeks. Groups of animals were necropsied at 2, 4, 5, 6, and 8 weeks after last exposure. Note the significant increase in MF in mice 2 through 6 weeks after exposure and in rats at 4 weeks after exposure. Exposed mice (●) and rats (◆); control animals (no markers); data points are means with error bars (n = 4–9 per group). An asterisk (∗) indicates a significant change compared with control values.

and only a 2.0-fold increase (p = 0.032) over control values was noted after 4 weeks of exposure. These results match well with results from a previous study using thymic T cells as a target for evaluating accumulation of *hprt* mutations in BD-exposed rodents (Meng et al. 1998a) (see Results section, Specific Aim 1).

The effect of elapsed time after BD exposures on *hprt* MFs in splenic T cells was evaluated following 4 weeks of exposure of mice and rats to 625 ppm BD (Experiment 2). In mice, mean [± SE] MFs were significantly increased 2 weeks after the last exposure ($p < 0.001$), continued to increase until maximal levels were reached 5 weeks after exposure (19.2 [± 4.8] × 10^{-6}; $p < 0.001$), and then gradually declined toward control values in some animals by 8 weeks after exposure (p = 0.075) (Figure 7). In BD-exposed rats, MFs were significantly increased only at 4 weeks after the last exposure

(5.4 [± 0.4] × 10^{-6}; $p = 0.032$), and they were just 2.0-fold higher than control values (Figure 7). The species-specific patterns in the changes in MFs shown in Figure 7 are similar to those observed previously following exposure of mice and rats to 1,250 ppm BD for 2 weeks (Meng et al. 1998a) (see Results section, Specific Aim 1).

The effects of concentration of BD exposure on *hprt* MFs in splenic T cells were evaluated in two experiments with different exposure durations (Figure 8). In the first dose-response study (Experiment 3A), animals were exposed to BD for 2 weeks and then necropsied 4 weeks after the last exposure. In mice, exposures to 20, 62.5, 625, or 1,250 (data from Meng et al. 1998a) ppm BD led to MF increases that were 2.0-fold ($p < 0.001$), 5.0-fold ($p < 0.001$), and 10.6-fold ($p < 0.001$) greater than those in control mice. The ability to detect a significant mutagenic effect in mice exposed to 20 ppm BD was enhanced by using a larger sample size for this treatment group and scheduling mice for necropsy at both 2 weeks (Table 3) and 4 weeks (Figure 8) after the last exposure. In exposed rats, the MFs in the 625 and 1,250 (data from Meng et al. 1998a) ppm BD dose groups were 1.7- ($p = 0.029$) and 3.5-fold ($p = 0.005$) greater than control values (Figure 8). In the second study (Experiment 3B), mice and rats were exposed to 20, 62.5, or 625 ppm BD for 4 weeks. Control and exposed animals were necropsied 4 weeks after the last exposure. In mice exposed to 20, 62.5, or 625 ppm BD, mean [± SE] MFs were 1.8-fold ($p < 0.001$), 2.7-fold ($p < 0.001$), and 10.4-fold ($p < 0.001$) above background level (1.8 [± 0.6] × 10^{-6}), respectively. In rats, significantly increased MFs were obtained only at 625 ppm BD (5.42 [± 0.42] × 10^{-6}, $p = 0.001$), and average MFs in exposed rats were just 2.0-fold higher ($p = 0.032$) than in control rats (2.70 [± 0.20] × 10^{-6}).

Effects of Elapsed Time After Exposure and Exposure Concentration on *hprt* Mutant Frequencies in T Cells from Spleen of Mice and Rats Exposed to BDO (Experiments 4 and 5)

To evaluate the effect of elapsed time after BDO exposure on *hprt* MFs in splenic T cells, necropsies were conducted at 1, 3, 5, 7, and 9 weeks after the last exposure for mice, and 1, 3, 5, and 7 weeks after the last exposure for rats following a 4-week exposure to 25 ppm BDO (Experiment 4; Figure 9). In BDO-exposed mice, significantly increased mean [± SE] MFs were found only at 7 weeks after exposure (4.7 [± 1.0] × 10^{-6}; $p < 0.001$). The average MF was 4.0-fold higher than in control mice (1.2 [± 0.4] × 10^{-6}). In rats, no significant increase in MFs was obtained at any time point, but a positive trend was observed at the last time point, 7 weeks after exposure ($p = 0.056$).

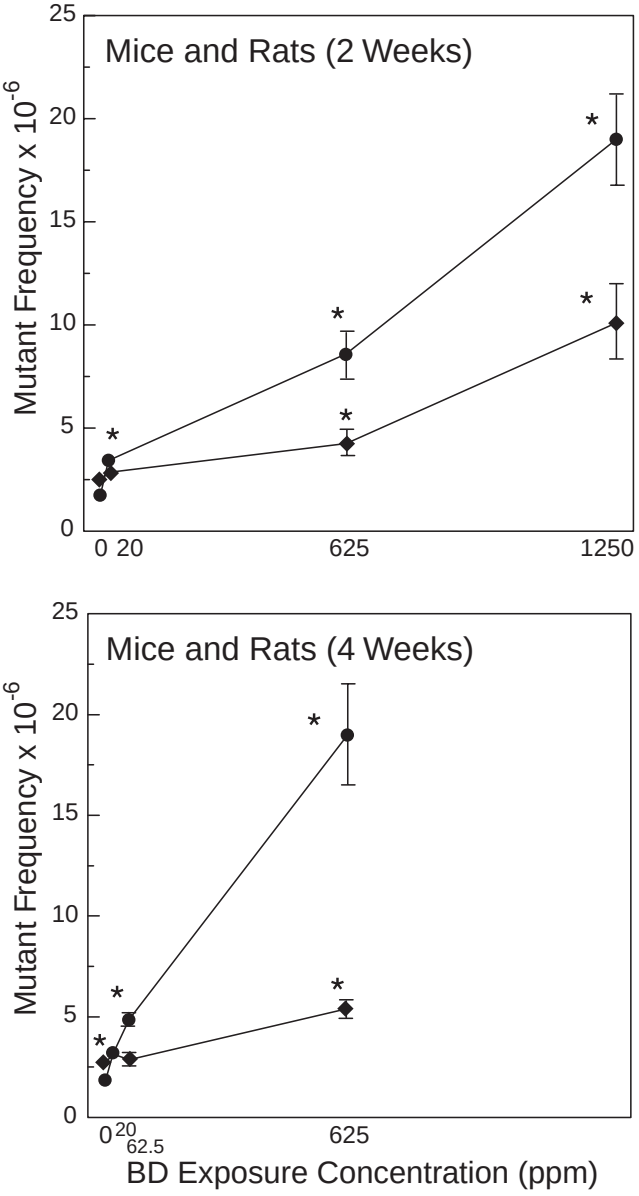

Figure 8. Dose responses for *hprt* mutant T cells from spleen of female B6C3F$_1$ mice and F344 rats following exposure to BD. Groups of mice and rats were exposed to either (top) 0, 20, 62.5 (data not shown), or 625 ppm BD for 2 weeks or (bottom) 0, 20, 62.5, or 625 ppm BD for 4 weeks. Data for animals exposed to 1,250 ppm BD (top) is from an earlier study (Meng et al. 1998a). Spleen was collected for T cell isolation and culture under selective conditions 4 weeks after the last exposure. Note the significant increase in MF in (1) mice at 20, 625, and 1,250 ppm BD for 2 weeks; (2) rats at 625 and 1,250 ppm BD for 2 weeks; (3) mice at 20, 62.5, and 625 ppm BD for 4 weeks; and (4) rats at 625 ppm BD for 4 weeks. Exposed mice (●) and rats (◆); data points are means with error bars ($n = 6$–9 per group). An asterisk (*) indicates a significant change compared with control values.

Table 3. Cloning Efficiency and *hprt* Mutant Frequency Data from Individual Mice Exposed to Air or 20 ppm BD[a]

Animal Number	Positive Wells of 192 Total Wells in Nonselection Plates	Cloning Efficiency (%)	Positive Wells / Total Wells in 6-Thioguanine Plates	Mutant Frequency ($\times 10^{-6}$ per Clonable Cell)
Control				
506	145	17.6	7 / 516	1.94
507	134	15.0	5 / 528	1.59
508	130	14.1	5 / 504	1.77
509	152	19.6	6 / 516	1.49
510	136	15.4	7 / 636	1.80
Mean ± SD		16.3 ± 2.2		1.72 ± 0.18
20 ppm BD				
551	149	18.7	18 / 516	4.75
552	135	15.2	16 / 864	3.07
553	126	13.3	13 / 624	3.96
554	118	11.9	9 / 769	2.48
555	151	19.3	15 / 684	3.65
556	141	16.6	9 / 576	2.37
557	139	16.1	12 / 624	3.02
558	132	14.5	8 / 384	3.63
559	129	13.9	13 / 528	4.48
Mean ± SD		15.5 ± 2.4		3.43 ± 0.81[b]

[a] Groups of control and exposed female B6C3F$_1$ mice (4–5 weeks old) were necropsied 2 weeks after a 2-week exposure period (6 hours/day, 5 days/week). T lymphocytes of spleen were separated as described in the Methods and Study Design section, primed with concanavalin A, and cultured in medium supplemented with rat T-STIM™ and IL-2. Cloning efficiencies and mutant frequencies were measured at 8–10 days after plating.

[b] Significant change from control group by the Mann-Whitney *U* statistic ($p < 0.001$).

To evaluate the effects of exposure concentration on *hprt* MFs in splenic T cells, mice and rats were exposed for 4 weeks to 0, 2.5, or 25 ppm BDO (Experiment 5; Figure 10). At 7 weeks after the last exposure, mice exposed to 2.5 or 25 ppm BDO had significant increases in mean [± SE] *hprt* MFs, 2.3-fold ($p = 0.006$) and 4.0-fold ($p < 0.001$) higher than control MFs (1.2 [± 0.4] × 10^{-6}), respectively. No significant dose-response relation was noted for MFs in rats exposed to BDO.

Effects of Elapsed Time After Exposure and Exposure Concentration on *hprt* Mutant Frequencies in T Cells from Spleen of Mice and Rats Exposed to BDO_2 (Experiments 6 and 7)

Change in *hprt* MFs in splenic T cells following BDO_2 exposure was defined in mice and rats exposed to 4 ppm BDO_2 for 4 weeks (Experiment 6; Figure 11). In exposed mice, mean [± SE] *hprt* MFs were significantly increased at 1 week after exposure ($p = 0.032$), gradually reached a maximum (4.3 [± 1.1] × 10^{-6}; $p = 0.041$) at 6 weeks after exposure, then declined toward background level at 9 weeks after exposure. In BDO_2-exposed rats, *hprt* MFs were significantly increased at 1 week after exposure ($p = 0.008$), increased in a linear fashion through 4 weeks after exposure to reach peak values at 6 weeks after exposure (10.4 [± 1.7] × 10^{-6}; $p = 0.009$). They then decreased toward control level at 9 weeks after exposure. Comparison of the resulting mutant T cell manifestation curves (Figure 11) shows that, in contrast to BD exposures (Figure 7), BDO_2 exposure induced a lower mutagenic response in mice than in rats.

The effects of BDO_2 exposure concentration on *hprt* MFs were evaluated in mice and rats exposed to BDO_2 for 4 weeks (Experiment 7). Exposure to either 2 or 4 ppm BDO_2 resulted in significant increases in MFs at 1, 2, 4, 6, and 7 weeks after exposure in both mice and rats (complete MF data are shown only for 4 ppm BDO_2 in Figure 11). At 4 weeks after exposure, MFs in mice exposed to 2 and 4 ppm BDO_2 were 2.5-fold ($p = 0.008$) and 2.8-fold ($p = 0.008$) higher than background (1.6 [± 0.2] × 10^{-6}), respectively (Figure 12). In rats, the MF increases in groups exposed to 2 or 4 ppm BDO_2 were 2.8-fold ($p = 0.004$) and 3.3-fold ($p = 0.008$) higher than control values (2.9 [± 0.2] × 10^{-6}). The shape of the dose-response curves in both mice and rats suggests that induction of *hprt* mutations approaches a plateau at the range of BDO_2 concentrations tested and that exposure to higher concentrations of BDO_2 would be unlikely to increase *hprt* MFs significantly.

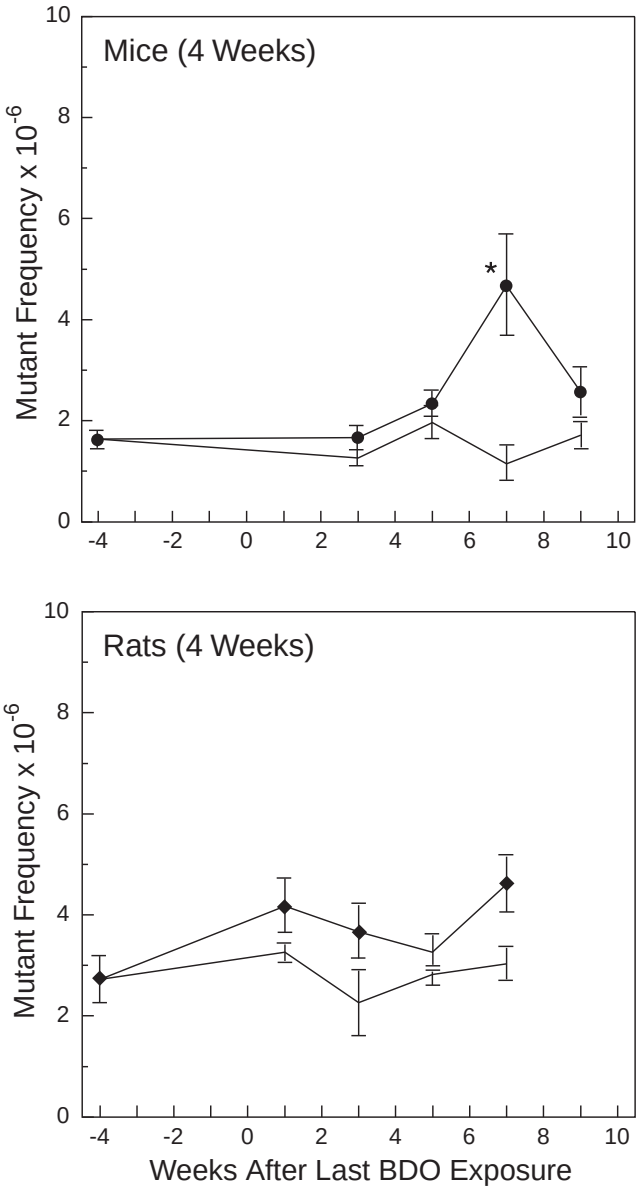

Figure 9. Relation between elapsed time since last BDO exposure and *hprt* MF in splenic T cells from female B6C3F$_1$ mice and F344 rats exposed to 0 or 25 ppm BDO for 4 weeks. Groups of mice were necropsied at 1 (data not shown), 3, 5, 7, and 9 weeks after exposure, and groups of rats were necropsied at 1, 3, 5, and 7 weeks after exposure. Note the significant increase in MF in mice only at 7 weeks after exposure. Exposed mice (●) and rats (◆); control animals (no markers); data points are means with error bars (n = 3–12 per group). An asterisk (∗) indicates a significant change compared with control values.

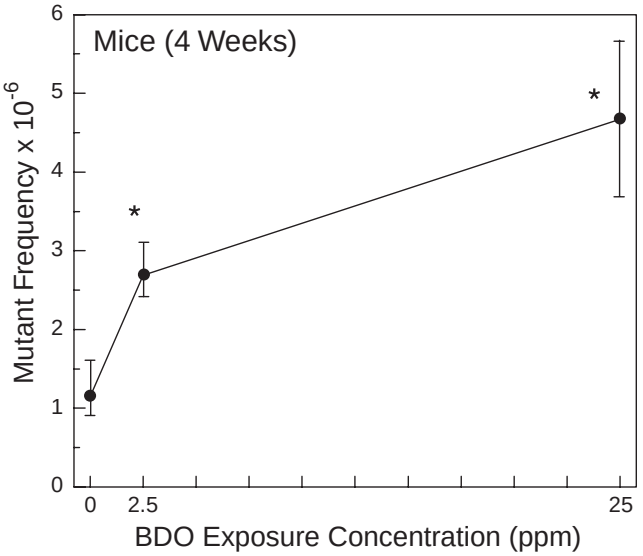

Figure 10. Dose responses for *hprt* mutant T cells from spleen of female B6C3F$_1$ mice 7 weeks after exposure to BDO. Groups of mice were exposed to 0, 2.5, or 25 ppm BDO for 4 weeks, and spleen was collected for T cell isolation and culture under selective conditions 4 weeks after last exposure. Mice exposed to both levels of BDO had significant increases in MF at 7 weeks after exposure. Data points are means with error bars (n = 3–12 per group). An asterisk (∗) indicates a significant change compared with control values.

Mutagenic Potency of BD, BDO, and BDO$_2$ Exposure in T Cells from Spleen of Mice and Rats

As noted above, the approach used to estimate mutagenic potency of BD, BDO, and BDO$_2$ exposure in mice and rats was based upon MF values in splenic T cells from all animals within a given experiment, including a limited number of MF values that were outliers within various control and exposure groups. These outliers usually had values that were higher by 10-fold or more than the corresponding data for a given group, and they may represent the occurrence of in vivo clonal amplifications of T cells carrying the same *hprt* mutation. In T cells isolated from spleen of animals exposed for 2 weeks to either 0 or 1,250 ppm BD (Specific Aim 1, Experiment 2), outliers in *hprt* MF values were found in 4/38 (10%) control mice, in 2/45 (4%) control rats, in 1/63 (< 2%) BD-exposed mice, and in 1/62 (< 2%) BD-exposed rats. In subsequent experiments in animals exposed for 4 weeks to BD (Specific Aim 2), outliers in *hprt* MF values were observed in 0/37 control mice, in 3/36 (8.3%) control rats, in 0/70 exposed mice, and in 1/70 (1.4%) exposed rats. In experiments with BDO, outliers in *hprt* MF values were observed in 0/26 control mice, in 0/24 control rats, in 2/48 (4.2%) exposed mice, and in 1/36 (2.8%) exposed rats. No outliers in *hprt* MF values were found in BDO$_2$-exposed or control animals. These data suggest that substantial clonal expansions of T cells occur at a low frequency in control and exposed rodents and have little impact upon the overall shapes of the mutant manifestation curves used to estimate mutagenic potencies in mice and rats (see Discussion and Conclusions section).

The effectiveness of applying a robust regression in minimizing the influence of outliers in MF values was tested

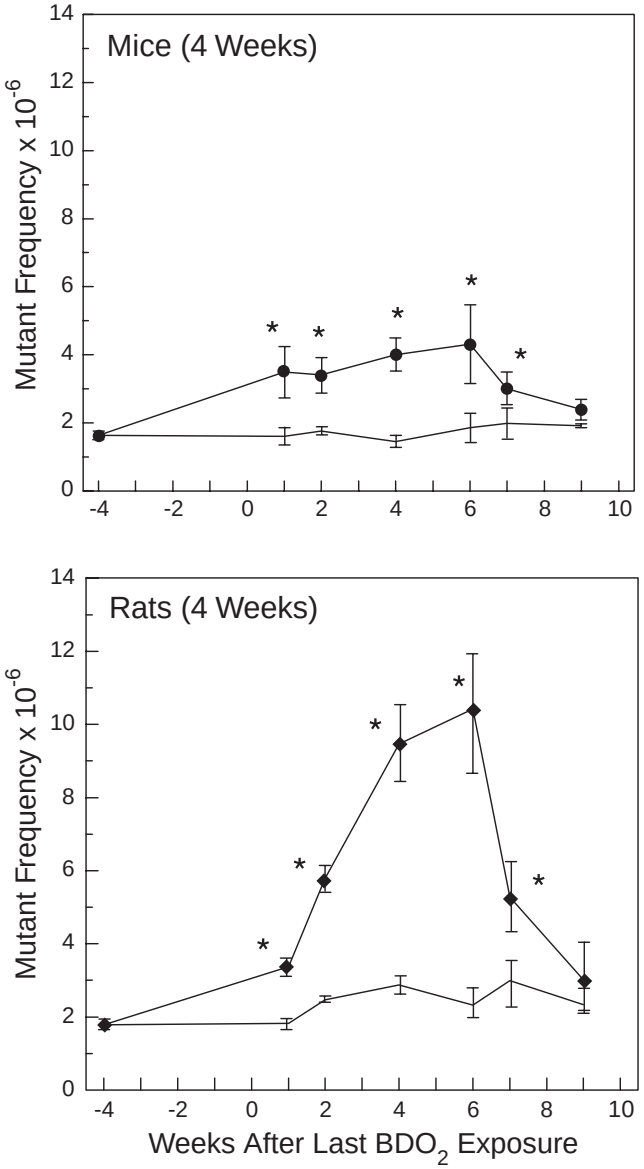

Figure 11. Relation between elapsed time after last BDO_2 exposure and *hprt* MF in splenic T cells from female $B6C3F_1$ mice and F344 rats exposed to 0 or 4 ppm BDO_2 for 4 weeks. Groups of animals were necropsied at 1, 2, 4, 6, 7, and 9 weeks after last exposure. Note the significant increase in MF in both mice and rats 1 through 7 weeks after exposure. Exposed mice (●) and rats (◆); control animals (no markers); data points are means with error bars (n = 4–6 per group). An asterisk (*) indicates a significant change compared with control values.

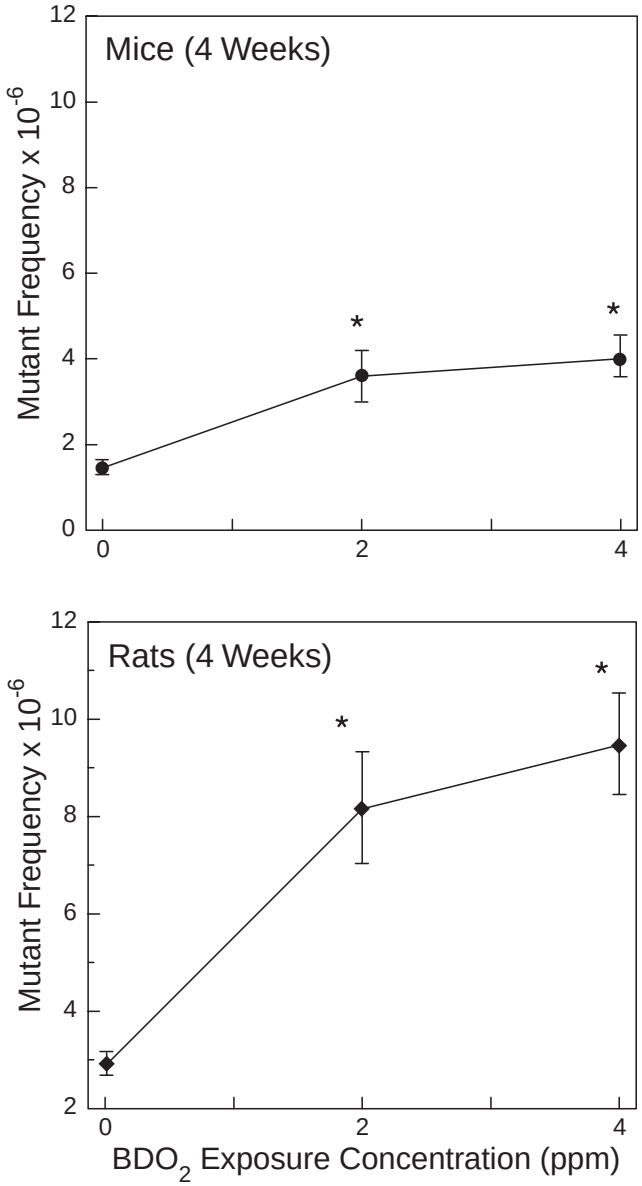

Figure 12. Dose responses for *hprt* mutant T cells from spleen of female $B6C3F_1$ mice and F344 rats following exposure to BDO_2. Groups of mice (●) and rats (◆) were exposed to 0, 2, or 4 ppm BDO_2 for 4 weeks. Spleen was collected for T cell isolation and culture under selective conditions 4 weeks after last exposure. Both mice and rats exposed to both levels of BDO_2 had significant increases in MFs at 1, 2, 4, 6, and 7 weeks after exposure. Data points are means with error bars; n = 4–6 per group. An asterisk (*) indicates a significant change compared with control values.

using MF data for splenic T cells from mice exposed for 2 weeks to 1,250 ppm BD (Specific Aim 1, Experiment 2). As mentioned above, this data set had 63 points including a single low outlier. In a quantification, 11 of the nonoutlier observations could be replaced with 8 standard deviation outliers without having the estimated response curve exceed the plotted 95% confidence interval. On this criterion, the procedure could be said to be robust to polynomial data sets containing up to 12/63, or 19%, severe outliers.

To quantify the mutagenic response to BD, BDO, and BDO_2 at the *hprt* locus in splenic T cells of mice and rats, curves were fitted to individual MF data with the difference in the areas under the curves for exposed and control animals representing the mutagenic potency of that compound in that species. In plotting the resulting curves

Table 4. Estimated Mutagenic Potencies[a] of BD, BDO, and BDO_2 on the Basis of *hprt* Mutant Frequencies in Splenic T cells of Control and Exposed Mice and Rats[b]

	BD		BDO		BDO_2	
	62.5 ppm	625 ppm	2.5 ppm	25 ppm	2 ppm	4 ppm
Mice	10.9	61.2[c]	3.9	8.8	11.2	12.7
Rats	NS[d]	7.2	NS	NS	20.9[c]	35.9[c]

[a] Mutagenic potency is a relative value that represents the number of mutant cells passing through the spleen over the period of time being evaluated; that is, mutagenic potency approximates the the [number of *hprt* mutants/number of wild-type cells] × [number of wild-type cells passing through the spleen/unit of time].

[b] Female $B6C3F_1$ mice and F344 rats (4–5 weeks old) were exposed to BD, BDO, or BDO_2 for 4 weeks by inhalation (6 hours/day, 5 days/week). Groups of control and exposed animals were necropsied at 2, 4, 5, 6, or 8 weeks after BD exposure; 1, 3, 5, 7, or 9 weeks after BDO exposure; or 1, 2, 4, 6, 7, or 9 weeks after BDO_2 exposure. Mutant frequencies for *hprt* were measured at each time point by T cell cloning assay (see the Methods and Study Design section), and curves were fitted to individual mutant frequency data (Figures 13–17). Mutagenic potency of each compound in each species was calculated by subtracting the area under the curve of control animals from the area under the curve of exposed animals. The mutagenic potencies of each compound at lower exposure levels were extrapolated from the data in animals exposed to higher doses of the same compound using the dose-response relation defined simultaneously.

[c] The 95% Monte Carlo CIs (for the mutagenic potencies) for mice (49.4, 74.7) and rats (4.2, 10.5) exposed to 625 ppm BD do not overlap, which demonstrates a significant increase in the mutagenic potency of BD in mice compared with rats. On the contrary, the lack of overlap in the CIs for BDO_2-exposed rats (16.3, 30.6 at 2 ppm, and 29.0, 46.9 at 4 ppm) and mice (7.4, 15.7 at 2 ppm, and 8.8, 17.1 at 4 ppm) shows a significant increase in the mutagenic potency of BDO_2 in rats compared with mice.

[d] NS = not significant.

(Figures 13–17), the same y-axis scale was used for each graph to facilitate visual comparison of the mutagenic potency of 62.5 or 625 ppm BD compared with the BDO and BDO_2 exposure concentrations that yielded similar blood levels of these metabolites. Table 4 presents the estimated mutagenic potencies in mice and rats exposed to the various levels of BD, BDO, and BDO_2. The mutagenic potencies of animals exposed to lower levels of chemicals were extrapolated from the data of animals exposed to higher doses of compounds using the dose-response relations defined simultaneously. The mutagenic potency of a 4-week inhalation exposure to 625 ppm BD in splenic T cells of mice (61.2) was 8.5-fold greater than in rats (7.2) (Figures 13 and 14, Table 4). The magnitude of this species difference in mutagenic potency is greater than that observed in mice and rats exposed to 1,250 ppm BD for 2 weeks (where the mutagenic potency was 4.4-fold greater in mice than in rats) (Meng et al. 1998a). The mutagenic potency of a 4-week exposure to 25 ppm BDO in splenic T cells of mice was 8.8 (Figure 15, Table 4), but no significant mutagenic response was noted in similarly exposed rats. In contrast to the results of the BD and BDO studies, the species differences in mutagenic responses to BDO_2 were reversed, with a 4-week exposure to 4 ppm BDO_2 leading to a mutagenic potency in splenic T cells that was 3.2 times greater in the rat than in the mouse (Figures 16 and 17, Table 4).

SPECIFIC AIM 3

Analysis of Mutations in *hprt* Exon 3 of T Cells from Spleen of Control Mice (Experiment 1)

A total of 394 *hprt*-negative T cell colonies from untreated or vehicle-treated $B6C3F_1$ mice were analyzed by PCR, DGGE, and DNA sequencing for the presence of mutations in exon 3. These mice were concurrent controls for exposures to various compounds, including BD, BDO, BDO_2, ethylene oxide, ethylnitrosourea, and cyclophosphamide (Walker et al. 1996, 1999; Meng et al. 1998a), and all mice were necropsied as young adults at 12 to 16 weeks of age (see footnotes to Table 6). Twenty *hprt* mutant colonies (5%) contained mutations in exon 3; these mutations included a combination of base substitutions (15/20, 75%), frameshifts (3/20, 15%), one small deletion (5%), and one complex mutation (5%). The 15 base substitutions occurred at 12 different sites, and they involved transitions and transversions at both G•C (11/20) and A•T (4/20) base pairs. One base substitution (G•C→T•A at base pair [bp] 152) was detected in three different mice, and another one (G•C→A•T at bp 168) was seen in two different mice. The frameshift mutations included two insertions and one deletion. Table 5 presents a summary of the classes of mutations found in exon 3 of control mice compared with those found in BD- and BDO_2-exposed mice, as well as BD- and BDO_2-exposed rats. Table 6 lists the types and locations of individual mutations that occurred in control mice.

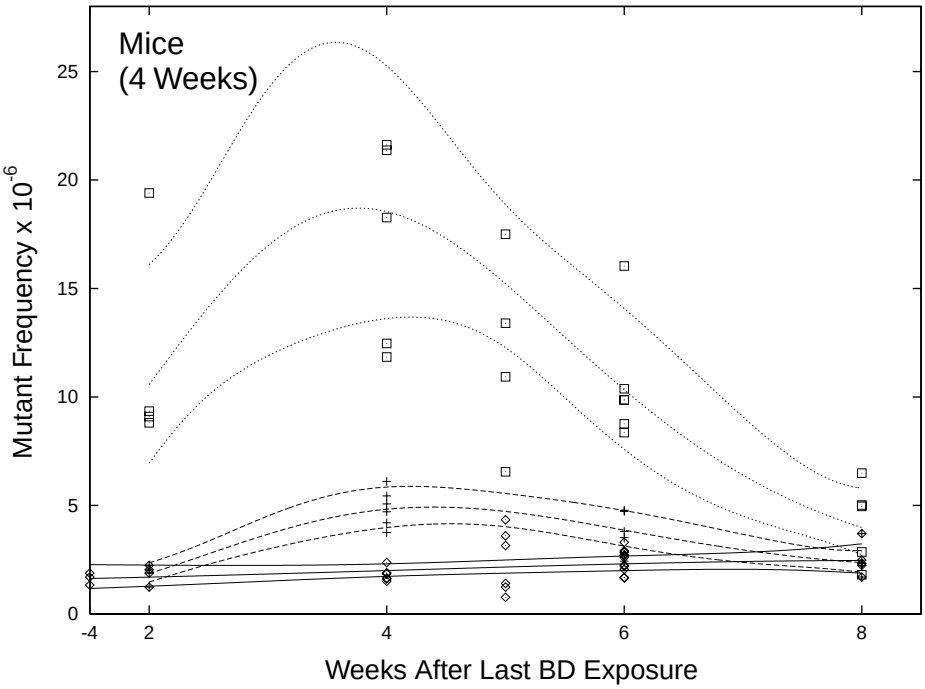

Figure 13. Curves of individual *hprt* MF data obtained following exposure of female B6C3F$_1$ mice to 0, 62.5, or 625 ppm BD for 4 weeks. Groups of mice were necropsied at 2, 4, 5, 6, or 8 weeks after last exposure. Data obtained between 2 and 8 weeks after exposure were used to derive robustly estimated polynomial models, with accompanying 95% CIs computed from the associated *t* distribution. Areas between estimated curves of exposed and control mice were used to compute the mutagenic potency for each exposure concentration of BD (see Table 2). For 0 ppm BD: curve and CIs —; data ◇. For 62.5 ppm BD: curve and CIs ----; data +. For 625 ppm BD: curve and CIs ·····; data ⊡.

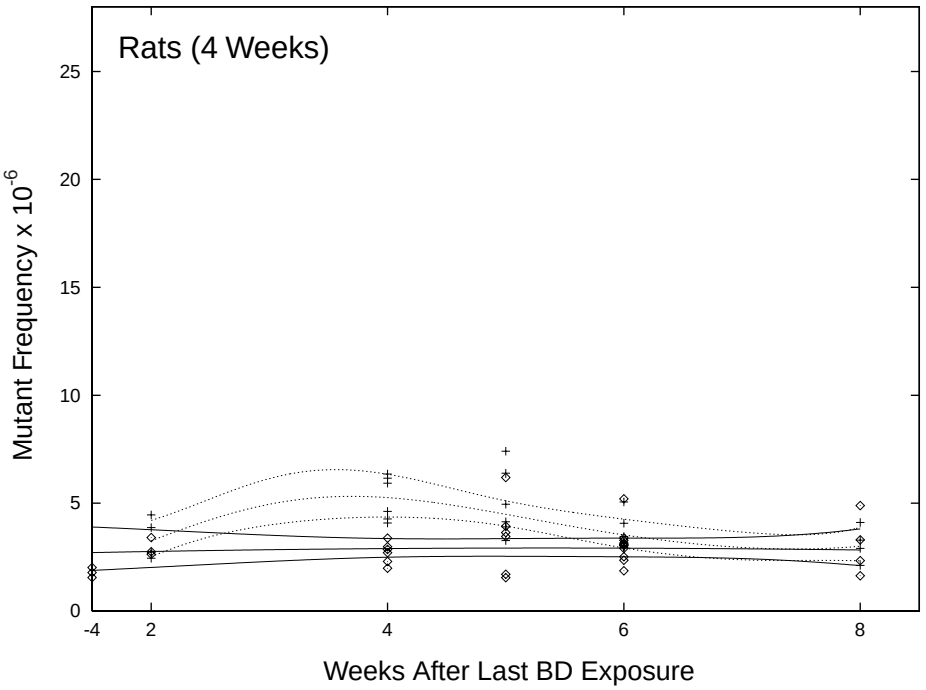

Figure 14. Curves of individual *hprt* MF data obtained following exposure of female F344 rats to 0 or 625 ppm BD for 4 weeks. Groups of rats were necropsied at 2, 4, 5, 6, or 8 weeks after last exposure. Data obtained between 2 and 8 weeks after exposure were used to derive robustly estimated polynomial models, with accompanying 95% CIs computed from the associated *t* distribution. Areas between estimated curves of exposed and control rats were used to compute the mutagenic potency of BD (see Table 2). For 0 ppm BD: curve and CIs —; data ◇. For 625 ppm BD: curve and CIs ----; data +.

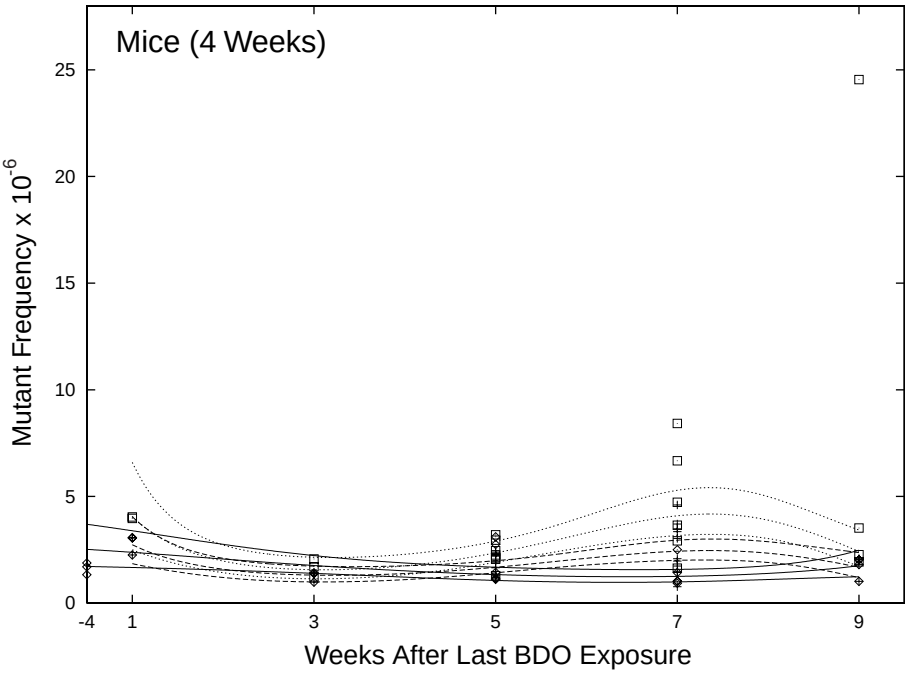

Figure 15. Curves of individual *hprt* MF data obtained following exposure of female B6C3F$_1$ mice to 0, 2.5, or 25 ppm BDO for 4 weeks. Groups of mice were necropsied at 1, 3, 5, 7, or 9 weeks after last exposure. Data obtained between 1 and 9 weeks after exposure were used to derive robustly estimated polynomial models, with accompanying 95% CIs computed from the associated *t* distribution. Areas between estimated curves of exposed and control mice were used to compute the mutagenic potency for each exposure concentration of BDO (see Table 2). For 0 ppm BDO: curve and CIs —; data ◇. For 2.5 ppm BDO: curve and CIs ----; data +. For 25 ppm BDO: curve and CIs ·····; data ⊡.

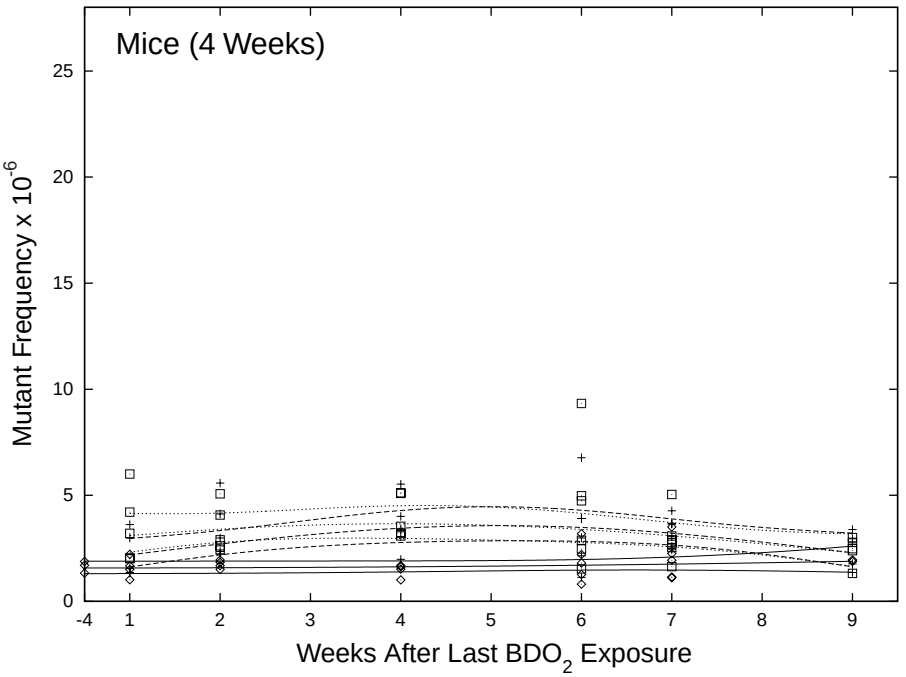

Figure 16. Curves of individual *hprt* MF data obtained following exposure of female B6C3F$_1$ mice to 0, 2, or 4 ppm BDO$_2$ for 4 weeks. Groups of mice were necropsied at 1, 2, 4, 6, 7, or 9 weeks after last exposure. Data obtained between 1 and 9 weeks after exposure were used to derive robustly estimated polynomial models, with accompanying 95% CIs computed from the associated *t* distribution. Areas between estimated curves of exposed and control mice were used to compute the mutagenic potency for each exposure concentration of BDO$_2$ (see Table 2). For 0 ppm BDO$_2$: curve and CIs —; data ◇. For 2 ppm BDO$_2$: curve and CIs ----; data +. For 4 ppm BDO$_2$: curve and CIs ·····; data ⊡.

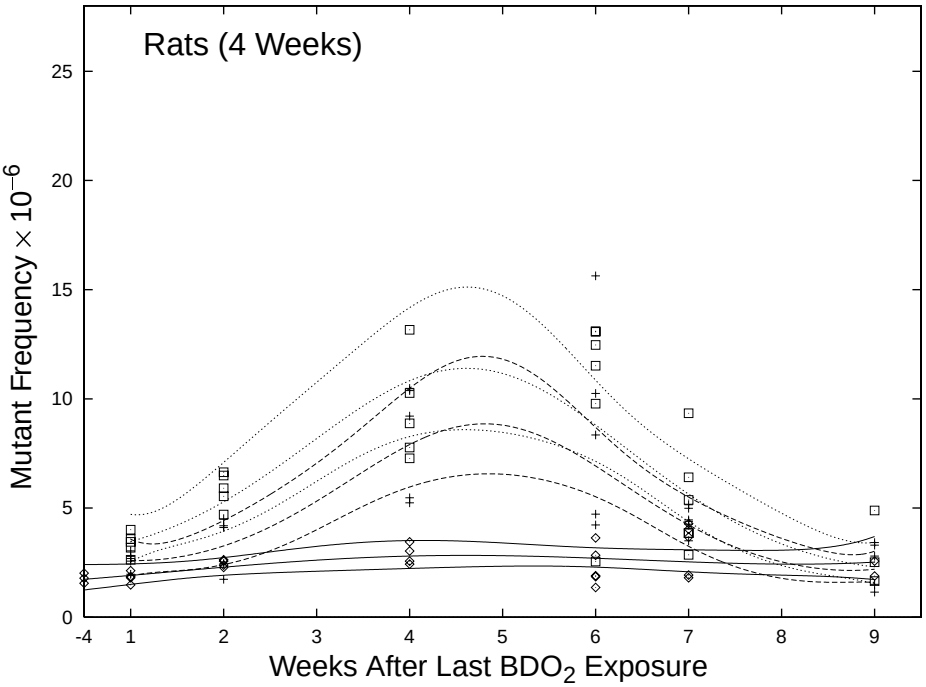

Figure 17. Curves of individual *hprt* MF data obtained following exposure of female F344 rats to 0, or 2, or 4 ppm BDO_2 for 4 weeks. Groups of rats were necropsied at 1, 2, 4, 6, 7, or 9 weeks after last exposure. Data obtained between 1 and 9 weeks after exposure were used to derive robustly estimated polynomial models, with accompanying 95% CIs computed from the associated *t* distribution. Areas between estimated curves of exposed and control rats were used to compute the mutagenic potency for each exposure concentration of BDO_2 (see Table 2). For 0 ppm BDO_2: curve and CIs —; data ◇. For 2 ppm BDO_2: curve and CIs ----; data +. For 4 ppm BDO_2: curve and CIs ·····; data ⊡.

Table 5. Summary of *hprt* Exon 3 Mutations in T Lymphocytes of Control $B6C3F_1$ Mice and $B6C3F_1$ Mice and F344 Rats Exposed to BD or BDO_2

		BD		BDO_2	
Type of Mutation	Control Mice	Mice	Rats	Mice	Rats
Base substitution					
G•C→A•T	7 (35%)[a]	6 (11%)	11 (19%)	28 (42%)	14 (28%)
A•T→G•C	2 (10%)	6 (11%)	4 (7%)	11 (17%)	6 (12%)
G•C→T•A	3 (15%)	5 (9%)	7 (12%)	2 (3%)	8 (16%)
G•C→C•G	1 (5%)	18 (32%)[a]	5 (9%)	8 (12%)	2 (4%)
A•T→C•G	1 (5%)	8 (14%)	9 (15%)	8 (12%)	8 (16%)
A•T→T•A	1 (5%)	4 (7%)	5 (9%)	3 (5%)	6 (12%)
Frameshift					
−1	1 (5%)	2 (4%)	1 (2%)	2 (3%)	4 (8%)
+1	2 (10%)	4 (7%)	11 (19%)	3 (5%)	0
Small deletion	1 (5%)	1 (2%)	6 (10%)	1 (2%)	2 (4%)
Small insertion	0	0	0	0	1 (2%)
Complex mutation	1 (5%)	2 (4%)	0	0	0
Total	20 (100%)	56 (100%)	59 (100%)	66 (100%)	51 (100%)

[a] Significant difference from controls ($p < 0.05$) with Fisher exact hypothesis test, as used by Carr and Gorelick (1996).

Table 6. Base Alterations in *hprt* Exon 3 of Splenic T Cells from Control B6C3F$_1$ Mice

Base[a] and Mutation[b]	Animal Number[c]																
	BDC-22	BDC-25	BDC-506	BDC-508	BDC-509	BDC-517	BDC-518	MS-15	MS-18	EC-1	EC-3	ETOC-15	ETOC-21	ETC-11	ETC-15	K-12	Total
145 C→T															1		1
151 C→T											1						1
152 G→T			1		1											1	3
165 G→A									1								1
168 G→A				1			1										2
179 A→G							1										1
207 G→A														1			1
213 C→G	1																1
252 A→G						1											1
288 T→G												1					1
299 T→A													1				1
311 G→A								1									1
135 –G										1							1
191 +A						1											1
243–248 –6 bp		1															1
289 +G												1					1
293 ATTT→CTCCC		1															1
Total	1	2	1	1	1	2	2	1	1	1	1	2	1	1	1	1	20

[a] Numbering of the *hprt* gene from controls, according to Melton et al. 1984.

[b] The mutation in the nontranscribed strand is reported.

[c] Control mice were necropsied at the following ages: prefixes MS, EC, ETC, and K, 12 weeks; prefixes BDC and ETOC, 12 to 16 weeks.

Analysis of Mutations in *hprt* Exon 3 of T Cells from Spleen of Mice Exposed to BD (Experiment 2)

A total of 712 *hprt* mutant T cell clones from mice exposed to 1,250 ppm BD for 2 weeks or to 625 ppm BD for 4 weeks were screened for mutations in exon 3. The *hprt* MFs in these BD-exposed mice were about 5- to 11-fold higher than those in control mice. Of the 712 mutants, 56 (8%) had mutations in exon 3. The majority of these mutations were base substitutions (47/56, 84%; 21 sites), whereas the rest were frameshift mutations (6/56, 11%; 6 sites), complex mutations (2/56, 4%), and one small deletion (2%) (Tables 5 and 7). The base substitutions were composed of transitions and transversions at both G•C (29/56, 52%; 11 sites) and A•T base pairs (18/56, 32%; 10 sites). Two different substitutions occurred at three of the mutant G•C base pairs (that is, bp 207, 211, and 219) and at two of the mutant A•T base pairs (that is, bp 217 and 299). The most common mutations in exon 3 were G•C→C•G transversions at bp 211 (found in four mice), G•C→C•G transversions at bp 212 (found in four mice), and A•T→C•G transversions at bp 216 (found in six mice).

Notably, 16/18 of the guanine bases involved in the G•C→C•G transversions found in BD-exposed mice were situated on the nontranscribed strand (Table 7), whereas the guanine in the only G•C→C•G transversion identified thus far in a control mouse occurred on the transcribed strand (Table 6). Also, all (8/8) of the thymine bases at risk in the A•T→C•G transversions of BD-exposed mice were located on the nontranscribed strand (Table 7). Remarkably, 38% (21/56) of the exon 3 mutations in BD-exposed mice were detected in a run of six guanines (bp 207–212); these mutations included one +G insertion, one –G deletion, and substitutions at four of the six guanine bases (Table 7). Finally, four sites in exon 3 (bp 151, 207, 299, and 311) were identified at which base substitutions occurred in both control and BD-exposed mice (among 17 and 30 mutant sites in each group, respectively), with identical mutations occurring at only two of these mutual sites (Tables 6 and 7).

Statistical analysis of the proportions of mutational types in control versus BD-exposed mice failed to demonstrate a significant difference in homogeneity ($p = 0.358$). However, when we used 20 independent mutations in controls and 49 independent mutations in BD-exposed mice as conditions of the analysis (as we did in the test for homogeneity of proportions) to assess the differences in individual base substitutions, the frequencies of G•C→A•T transitions ($p = 0.035$) and G•C→C•G transversions ($p < 0.05$) were significantly different (Table 5). The five *hprt* mutant clones from one BD-exposed mouse (Table 7, animal 609) had G•C→C•G transversions at bp 211, which may represent a clonal expansion of a single mutation (see Discussion and Conclusions section). Nevertheless, the frequency of this mutational type would remain significantly elevated in BD-exposed mice even if these duplicate mutations in mouse 609 were considered mutant siblings and counted as a single mutation. Finally, the mutant fraction (that is, [observed MF accounted for by the percentage of mutant clones with mutations in exon 3] × [average percentage of total exon 3 mutants for a class of mutation]) can be used to estimate the induced MF within each class of mutation (that is, [mutant fraction in BD-exposed mice] – [mutant fraction in control mice]). The resulting mutant fraction data for control and BD-exposed mice suggest that BD induces a variety of base substitutions and other mutations in *hprt* exon 3 (see details in Meng et al. 2000). Notably, extending this analysis to the fraction of mutations in the run of six guanines in exon 3 indicated that BD exposure of mice was associated with an 80-fold increase in the occurrence of mutations at bp 207–212 compared with control mice (Meng et al. 2000).

Analysis of Mutations in *hprt* Exon 3 of T Cells from Spleen of Rats Exposed to BD (Experiment 3)

A total of 1,168 *hprt* mutant clones collected from F344 rats exposed to 1,250 ppm BD for 2 weeks or to 625 ppm BD for 4 weeks were evaluated for mutations in exon 3. The *hprt* MFs in these BD-exposed rats were 2- to 3-fold higher than the MF values in control rats. Analysis and sequencing by DGGE revealed that 59 T cell mutants (5%) contained mutations in exon 3; these mutations included a combination of base substitutions (41/59, 70%; 24 sites), frameshifts (12/59, 20%; 6 sites), and small deletions (6/59, 10%; 6 sites) (Tables 5 and 8). Transitions and transversions were observed at both G•C (23/59, 39%; 15 sites) and A•T (18/59, 31%; 9 sites) base pairs. Two different base substitutions occurred at two of the mutant A•T base pairs (that is, bp 215 and 299) and at one of the mutant G•C base pairs (that is, bp 208). The most common base substitution was an A•T→C•G transversion at bp 216 (found in 4 rats). Moreover, 7/9 of the thymine bases at risk in the A•T→C•G transversions in BD-exposed rats were located on the nontranscribed strand. Among the frameshift mutations, three different insertions were each observed in more than one rat (that is, +T at bp 140 in two rats, +G at bp 207–212 in two rats, and +T at bp 299/300 in three rats). Of the exon 3 mutations detected in BD-exposed rats, 13/59 (22%) occurred at a run of six guanines (bp 207–212), and these included the +G insertions and the base substitutions at five of the six guanines. Interestingly, the set of deletions found in BD-exposed rats, included two deletions that were substantially larger (–37 and –25 bp) than those usually identified by DGGE. Finally, 12 sites with base substitutions were the same in BD-exposed mice (among 21 total sites with substitutions) and rats (among 24 total sites with substitutions), and nine of these shared sites had identical mutations (Tables 7 and 8).

Table 7. Base Alterations in *hprt* Exon 3 of Splenic T Cells from B6C3F$_1$ Mice Exposed to BD

Base[a] and Mutation[b]	Animal Number																		
	86	88	89	93	97	103	107	108	114	117	118	119	605	606	607	608	609	610	Total
151 C → T												1							1
164 A→T																		1	1
171 G→A													1	1					2
190 G→T									1										1
197 G→A																		1	1
204 C→G														1			1		2
206 A→G				1															1
207 G→T										1									1
207 G→C														1					1
208 G→T													1						1
211 G→T				1															1
211 G→C													2	1		2	5		10
212 G→C													1	2		1	1		5
214 T→G															1				1
216 T→G			1	1				1			1				1		1		6
217 A→G			1																1
217 A→T													1						1
219 G→A	1																		1
219 G→T																	1		1
221 T→C			1			1													2
223 T→C											1								1
228 T→A								1											1
230 A→T														1					1
299 T→C		1																	1
299 T→G																		1	1
311 G→A																	1		1
187 +G	1																		1
207–212 +G										1									1
207–212 –G							1												1
214–220 –7 bp																	1		1
228 +A							1												1
228–229 TG →C								1											1
229 +A																	1		1
239–244 ATTACA →CT																	1		1
247–249 –A					1														1
Total	2	1	3	3	1	1	2	3	1	2	2	1	6	7	2	3	13	3	56

[a] Numbering of the *hprt* gene according to Melton et al. 1984.

[b] The mutation in the nontranscribed strand is reported.

Table 8. Base Alterations in *hprt* Exon 3 of Splenic T Cells from F344 Rats Exposed to BD

Base[a] and Mutation[b]	Animal Number																							
	287	288	289	291	292	293	294	295	297	298	300	301	302	303	304	307	312	317	320	822	823	824	825	Total
151 C→T													1											1
154 G→A		1						1				1		1										4
156 T→A							1																	1
162 G→C	1																							1
164 A→T																							3	3
165 G→T				1																				1
190 G→C																						1		1
195 C→A															1									1
202 C→A															1									1
207 G→A								1																1
208 G→A																2	1			1				4
208 G→C															1									1
210 G→T																				1				1
211 G→C						1																		1
212 G→T					1																			1
215 A→C							2																	2
215 A→G										1														1
216 T→G	2		1																	1		1		5
219 G→T																					1		1	2
221 T→C																					1			1
277 A→G																							1	1
287 C→G									1															1
296 T→G																1								1
298 A→T																					1			1
299 T→C	1																							1
299 T→G																				1				1
311 G→A								1																1
140 +T											1							1						2
175–180 –6 bp				1																				1
227–237 –8 bp[c]																		1						1
207–212 +G							2												2					4
219–255 –37 bp																						1		1
220 or 221 +T																1								1
243 –C					1																			1
244–249 –6 bp																				1				1
283–308 –25 bp							1																	1
294–297 +T																		1						1
299 or 300 +T															1			1			1			3
300–303 or 301–304–4 bp											1													1
Total	4	1	1	2	2	1	6	3	1	1	2	1	1	1	4	4	1	4	2	5	4	3	5	59

[a] Numbering of the *hprt* gene according to Melton et al. 1984.

[b] The mutation in the nontranscribed strand is reported.

[c] The range bp 227–237 contains eight possible sites for this 8-bp deletion.

Analysis of Mutations in *hprt* Exon 3 of T Cells from Spleen of Mice Exposed to BDO_2 (Experiment 4)

A total of 642 *hprt* mutant colonies from mice exposed to 4 ppm BDO_2 for 4 weeks were assessed for mutations in exon 3. The *hprt* MFs in these BDO_2-exposed mice averaged 2.7-fold (ranging from 2- to 3-fold) higher than those measured in control mice. Of the 642 mutants, 66 (10%) were found to contain mutations in exon 3; these mutations included base substitutions (60/66, 91%; 22 sites), frameshift mutations (5/66, 7.5%; 5 sites), and one small deletion (1.5%) (Tables 5 and 9). The base substitutions consisted of transitions and transversions at both G•C (38/66, 58%; 13 sites) and A•T (22/66, 33%; 9 sites) base pairs. Two different substitutions were found at two of the mutant G•C base pairs (that is, bp 166 and 211). The most frequently occurring exon 3 mutations in BDO_2-exposed mice were G•C→A•T transitions at bp 208 (found a total of 12 times in eight different mice), G•C→C•G transversions at bp 211 (found a total of four times in three different mice), and A•T→C•G transversions at bp 216 (found a total of seven times in six different mice).

The frequency of the G•C→C•G and A•T→C•G transversions in BDO_2-exposed mice appeared to be increased when compared with transversions in control mice; however, due to the small sample size of control mutations, the increases were not significant. Notably, all 28 of the guanine bases involved in the G•C→A•T transitions found in BDO_2-exposed mice were situated on the nontranscribed strand (Table 9), whereas in control mice the guanines in the analogous transitions occurred on both the nontranscribed (5/7, 71%) and transcribed strands (2/7, 29%) (Table 6). Likewise, in BDO_2-exposed mice, all of the guanine bases at risk in the G•C→C•G transversions and all of the thymine bases at risk in the A•T→C•G transversions were located on the transcribed strand.

Remarkably, one third (22/66) of the exon 3 mutations occurring in BDO_2-exposed mice were found in the run of six guanines at bp 207–212. These mutations included a single –G deletion and base substitutions at five of the six guanines; however, no +G insertions were found in this same stretch of guanines in BDO_2-exposed mice. Four sites in exon 3 (bp 168, 207, 252, and 299) were identified at which base substitutions occurred in both control and BD-exposed mice (among 17 and 28 mutant sites in each group, respectively). Identical mutations were found at two of these four sites (Tables 6 and 9). Finally, 12 sites with base substitutions were common to BD-exposed mice (among 21 total sites with substitutions) and BDO_2-exposed mice (among 22 total sites with substitutions); eight of these mutual sites had identical mutations (Tables 7 and 9).

Statistical analyses failed to demonstrate a significant difference in the spectral data between control and BDO_2-exposed mice; but the mutant fraction estimates for control and exposed animals suggested that BDO_2 exposure was associated with induction of several types of point mutations (Meng et al. 2000). Notably, BDO_2 exposure was associated with a 30-fold increase in the occurrence of mutations at bp 207–212 compared with control mice. Furthermore, when the average increases in *hprt* MFs in BDO_2-exposed (~2.7-fold over background) and BD-exposed (~7.2-fold over background) mice were considered, the frequency of mutations at bp 207–212 in BDO_2- and BD-exposed mice appeared to be consistent.

Analysis of Mutations in *hprt* Exon 3 of T Cells from Spleen of Rats Exposed to BDO_2 (Experiment 5)

A total of 732 *hprt* mutant clones collected from rats exposed to 4 ppm BDO_2 for 4 weeks or to 5 ppm BDO_2 for 6 weeks (Meng et al. 1997) (Specific Aim 2) were examined for mutations in exon 3. The *hprt* MFs in BDO_2-exposed rats were 3- to 4.5-fold higher than those in the control rats. Analysis and sequencing by DGGE showed that 51 T cell mutants (7%) had mutations in exon 3; these mutations included 44 base substitutions (86%; 24 sites), 4 frameshifts (8%; 4 sites), 2 small deletions (4%; 2 sites), and 1 small insertion of 8 bp (2%) (Tables 5 and 10). Transitions and transversions were found at both G•C (24/51, 47%; 13 sites) and A•T base pairs (20/51, 37%; 11 sites). Two different base substitutions occurred at two of the mutant G•C base pairs (namely, bp 190 and 308). The most common substitution was an A•T→C•G transversion at bp 216, which was observed five times in three different rats. Furthermore, seven of the eight thymine bases involved in A•T→C•G transversions were located on the nontranscribed strand. Only five mutations were found in the six consecutive guanines of exon 3 (bp 207–212); however, these mutations (one –G deletion and three substitutions) did not include a +G insertion. The two deletions detected in BDO_2-exposed rats were relatively sizable (–24 and –74 bp) compared with deletions previously identified with DGGE techniques.

Finally, eight sites with base substitutions were common to both BD-exposed and BDO_2-exposed rats (among 24 total base-substitution sites in each group), and all but one of these mutual sites had identical mutations (Tables 8 and 10). In contrast, just five sites were identified at which base substitutions occurred in both BDO_2-exposed rats and mice (among 24 and 22 total base substitution sites in each species, respectively), and identical mutations occurred at three of the five shared sites (Tables 7 and 10).

Table 9. Base Alterations in *hprt* Exon 3 of Splenic T Cells from B6C3F$_1$ Mice Exposed to BDO$_2$

Base[a] and Mutation[b]	Animal Number																																					
	29	30	31	32	33	34	35	37	38	39	40	41	42	43	44	45	48	50	55	56	63	64	65	67	68	69	70	71	72	73	74	77	81	Total
143 G→C																	2	1																3
166 G→T																							1											1
166 G→A												1															1							2
168 G→A																							1	1										2
173 G→A							1																											1
197 G→A					1	1																												2
206 A→T										1																1								2
207 G→T																			1															1
208 G→A											3			2	1	1								1				1			1	2		12
210 G→A										1																								1
211 G→C		1						2																			1							4
211 G→A																											2							2
212 G→A																														1				1
214 T→A								1																										1
216 T→G											1	1								1							1		2		1			7
219 G→A	1																							1	1								1	4
220 T→C																		1										1						2
221 T→C				1																														1
223 T→C							2						1																		2			5
252 A→G			1																															1
260 G→A		1																																1
273 A→G			1			1																												2
299 T→G																					1													1
302 G→C																						1												1
158 +A																											1							1
187 +A															1																			1
207–212 –G									1																									1
215–216 –AT or 216–217 –TA									1																									1
219 –G													1																					1
222 +A																											1							1
Total	1	2	2	1	1	2	3	3	2	2	4	2	2	2	2	1	2	2	1	1	1	1	2	3	1	1	7	2	2	1	4	2	1	66

[a] Numbering of the *hprt* gene according to Melton et al. 1984.

[b] The mutation in the nontranscribed strand is reported.

Table 10. Base Alterations in *hprt* Exon 3 of Splenic T Cells from F344 Rats Exposed to BDO_2

Base[a] and Mutation[b]	Animal Number											
	137	138	139	143	144	145	173	174	175	176	177	Total
146 T→C						1						1
148 G→C								1				1
152 G→T							1					1
154 G→A								1				1
164 A→T									3	1	1	5
168 G→T							1					1
179 A→G										1		1
190 G→A		1										1
190 G→C											1	1
191 C→A				1								1
194 T→C		1										1
203 T→G	1	1										2
204 G→A							2		1			3
205 A→T		1										1
208 G→T		1										1
208 G→A										2	1	3
216 T→G	3	1	1									5
217 A→C			1									1
219 G→T		2	1						1			4
221 T→C									1			1
274 T→C										1		1
281 C→T								1				1
285 G→A					1							1
296 T→C				1								1
304 C→T				1								1
311 G→A								2	1			3
174–248 or 175–249 –74 bp			1									1
175 or 176 –G									1			1
199 –G							1					1
201 –G							1					1
207–212 –G							1					1
280–303 or 281–304 –24 bp										1		1
283 +8 bp		1										1
Total	4	9	4	3	1	1	7	5	8	6	3	51

[a] Numbering of the *hprt* gene according to Melton et al. 1984.

[b] The mutation in the nontranscribed strand is reported.

SPECIFIC AIM 4

Analysis of Mutations in the *hprt* Gene of T Cells from Spleen of Control and BD-Exposed Mice

Molecular analyses for mutations were performed on propagated *hprt* mutant clones from groups of control and BD-exposed mice with mean [± SE] MFs of 2.6 [± 0.4] × 10^{-6} and 10.7 [± 3.1] × 10^{-6}, respectively. All expanded mutant T cell clones from control and BD-exposed mice were initially analyzed by RT-PCR to produce *hprt* cDNA for sequencing. Mutant clones that did not produce an RT-PCR product for *hprt* were then analyzed for mRNA by RT-PCR amplification of the β-*actin* gene. Evidence of mRNA from *hprt* or β-*actin* cDNAs was found for 42/47 and 63/72 expanded clones from control and BD-exposed mice, respectively (Tables 11–13).

Analysis of the 42 spontaneous *hprt* mutant clones yielding cDNAs revealed 25 (60%) point mutations, 6 (14%) single-base insertions or deletions, 6 (14%) single-exon deletions, and 5 (12%) large deletions encompassing all of the *hprt* locus (Tables 11 and 12). Of the base substitutions, 60% (15/25) were found at A•T base pairs, with the majority of these mutations occurring at the adenine on the transcribed strand (12/15, 80%). However, the proportions of transitions (12/42) and transversions (13/42) were nearly equal. Specific base substitutions at four different sites (that is, bp 29, 299, 389, and 464) were observed in more than one animal (Table 12).

In BD-exposed mice, the mutations in 63 *hprt* mutant clones yielding cDNAs comprised 18 (29%) point mutations, 12 (19%) single-base insertions or deletions, 14 (21%) deletions of one to several exons, 19 (30%) large deletions, and 1 complex mutation (Tables 11 and 13). Of the base substitutions, 55% (10/18) were found at G•C base pairs, with the majority of these mutations occurring at the guanine on the nontranscribed strand (9/10, 90%). However, the proportions of transitions (10/64) and transversions (8/64) were similar. The most common base substitution was a G•C→A•T transition at bp 208, observed in three of five mice. Finally, A•T→T•A transversions at bp 299 were the only point mutations found in *hprt* cDNA of both control and BD-exposed mice (Tables 12 and 13).

Statistical analysis (chi-square distribution on 3 degrees of freedom) was done on the percentage of each mutational type among all mutations defined in control and BD-exposed mice to assess differences in mutational spectra (Table 11). The percentage of base substitutions in controls (25/42, 60%) was significantly greater ($p = 0.008$) than that observed in BD-exposed mice (18/63, 29%). In contrast, the percentage of large deletions in BD-exposed mice (19/63, 30%) was significantly greater ($p = 0.016$) than the percentage among background mutants (5/42, 12%). Finally, it should be noted that cells of female mice have two *hprt* alleles, and PCR methods that are both quantitative and specific for *hprt* exons of the mouse have not yet been developed. Thus, no method currently exists to determine if the deletion mutations found in *hprt* cDNA of some mutant T cell clones from control and BD-exposed mice were the result of mutations in splice donor and acceptor sites that could lead to aberrant splicing of *hprt* mRNA.

Table 11. Summary of *hprt* Mutations in Expanded T Cell Clones from Control and BD-Exposed Mice

Type of Mutation	Control Mice	BD-Exposed Mice
Base substitution		
G•C→A•T	6 (14.3%)	8 (12.7%)
A•T→G•C	6 (14.3%)	2 (3.2%)
G•C→T•A	2 (4.8%)	1 (1.6%)
G•C→C•G	2 (4.8%)	1 (1.6%)
A•T→C•G	0	3 (4.8%)
A•T→T•A	9 (21.4%)	3 (4.8%)
Total substitutions	25[a]	18
Insertion	4 (9.5%)	9 (14%)
Single base-pair deletion	2 (4.8%)	3 (4.8%)
Exon deletion	6 (11.9%)	13 (20.6%)
Large deletion	5 (12%)	19 (30.2%)[b]
Complex mutation	0	1 (1.6%)
Total mutations	42 (100%)	63 (100%)

[a] $p = 0.008$, chi-square distribution on 3 degrees of freedom.

[b] $p = 0.016$, chi-square distribution on 3 degrees of freedom.

Table 12. DNA Sequence Analysis of Expanded *hprt* Mutant T Cell Clones Isolated from Control Mice

Animal Number and Mutant Clone Number	Sequence Alteration[a]	Position (bp)[b]	Predicted Amino Acid Change	Target Sequence
Base Substitutions				
202-6	T→C	29	Ile→Pro	GTG ATT AGC
203-1	T→C	29	Ile→Pro	GTG ATT AGC
204-2	A→T	98	Glu→Val	TTG GAA AAA
205-8	A→T	99	Glu→Asp	TTG GAA AAA
204-5	T→C	170	Met→Pro	GAG ATG GGA
202-7	T→C	194	Leu→Thr	GCC CTC TGT
205-1	T→C	295	Phe→Leu	GAT TTT ATC
203-2	T→A	299	Ile→Asn	TTT ATC AGA
203-4	T→A	299	Ile→Asn	TTT ATC AGA
205-10	T→A	299	Ile→Asn	TTT ATC AGA
205-17	T→A	299	Ile→Asn	TTT ATC AGA
203-3	T→A	389	Val→Asp	AAT GTC TTG
205-14	T→A	389	Val→Asp	AAT GTC TTG
206-1	T→A	542	Phe→Tyr	GGA TTT GAA
202-8	A→G	611	His→Arg	AAT CAC GTT
204-1	G→A	32	Ser→Asn	ATT AGC GAT
206-5	C→T	193	Leu→Phe	GCC CTC TGT
203-5	G→C	285	Met→Ile	CCT ATG ACT
205-18	C→T	464	Thr→Leu	AGC CCC AAA
206-7	C→T	464	Thr→Leu	AGC CCC AAA
206-11	G→C	514	Val→Leu	AGT GTT GGA
205-19	C→A	522	Tyr→term	GGA TAC AGG
204-7	G→A	553	Asp→Asn	CCA GAC AAG
206-3	C→A	610	His→Asn	AAT CAC GTT
206-6	C→T	632	Pro→Ile	AGT GAA ACT
Insertions				
205-33	+A	277–278	Frameshift	TCC AA^ATT CCT
202-9	+C	482–483	Frameshift	GTT GC^CA AGC
205-35	+G	538–540	Frameshift	GTT GGG^A TTT
206-4	+C	579	Frameshift	GCC CTC^T GAC
Deletions				
205-30	–G	461	Frameshift	TAC AGC CCC
206-8	–C	506	Frameshift	ACC TCT CGA
203-10	–Exon 2	28–134	Frameshift	
204-6	–Exon 5	385–402	In-frame deletion	
202-4	–Exon 6	403–485	Frameshift	
205-11	–Exon 6	403–485	Frameshift	
203-11	–Exon 8	533–609	Frameshift	
205-5	–Exon 8	533–609	Frameshift	
202-5	Large deletion			
205-19	Large deletion			
205-20	Large deletion			
205-21	Large deletion			
206-2	Large deletion			

[a] The mutation in the nontranscribed strand is reported.

[b] Numbering of the *hprt* gene according to Melton et al. 1984.

Table 13. DNA Sequence Analysis of Expanded *hprt* Mutant T Cell Clones Isolated from BD-Exposed Mice

Animal Number and Mutant Clone Number	Sequence Alternation[a]	Position (bp)[b]	Predicted Amino Acid Change	Target Sequence
Base Substitutions				
625-8	T→A	23	Val→Asp	AGC GTC GTG
625-5	T→C	221	Phe→Ala	AAG TTC TTT
624-3	A→C	277	Ile→Leu	TCC ATT CCT
625-10	T→A	299	Ile→Asn	TTT ATC AGA
625-13	T→A	299	Ile→Asn	TTT ATC AGA
624-6	T→C	395	Ile→Pro	TTG ATT GTT
624-5	A→C	466	Lys→Gln	CCC AAA ATG
623-11	A→C	511	Ser→Arg	CGA AGT GTT
625-12	G→C	25	Val→Leu	GTC GTG ATT
622-11	G→A	58	Asp→Asn	CTA GAT TTG
624-10	G→A	58	Asp→Asn	CTA GAT TTG
622-1	G→T	58	Asp→Tyr	CTA GAT TTG
622-6	C→T	145	Leu→Phe	AGA CTT GCT
622-8	G→A	208	Gly→Arg	AAG GGG GGC
623-12	G→A	208	Gly→Arg	AAG GGG GGC
625-15	G→A	208	Gly→Arg	AAG GGG GGC
621-8	G→A	529	Asp→Asn	CCA GAC TTT
621-10	G→A	529	Asp→Asn	CCA GAC TTT
Insertions				
624-4	+T	32	Frameshift	ATT ATGC GAT
625-19	+G	58	Frameshift	CTA GGAT TTG
625-17	+A	76/77	Frameshift	CCT AAAT CAT
625-16	+G	181	Frameshift	CAT GCAC ATT
621-5	+C	272	Frameshift	GAT ACGA TCC
622-11	+G	333–337	Frameshift	ACG GGGG GAC
623-7	+G	333–337	Frameshift	ACG GGGG GAC
623-8	+C	469	Frameshift	AAA CATG GTT
623-13	+C	496	Frameshift	GTG CAAA AGG
Deletions				
621-6	–G	14	Frameshift	CGC AGT CCC
624-9	–C	503/504	Frameshift	AGG ACC TCT
623-6	–G	601	Frameshift	AGC GAT TTG
622-10	–Exon 2	28–134	Frameshift	
625-2	–Exon 2	28–134	Frameshift	
625-3	–Exon 5	385–402	In-frame deletion	
621-2	–Exon 6	403–485	Frameshift	
621-3	–Exon 7	486–532	Frameshift	
622-9	–Exon 7	486–532	Frameshift	
623-5	–Exon 7	486–532	Frameshift	
621-1	–Exon 8	533–609	Frameshift	
625-20	–Exons 2–3	28–318	In-frame deletion	
623-1	–Exons 2–4	28–384	In-frame deletion	
623-9	–Exons 2–4	28–384	In-frame deletion	
623-2	–Exons 2–4	28–384	In-frame deletion	

Table continues next page

[a] The mutation in the nontranscribed strand is reported.

[b] Numbering of the *hprt* gene according to Melton et al. 1984.

Table 13. DNA Sequence Analysis of Expanded *hprt* Mutant T Cell Clones Isolated from BD-Exposed Mice *(Continued)*

Animal Number and Mutant Clone Number	Sequence Alternation[a]	Position (bp)[b]	Predicted Amino Acid Change	Target Sequence
Deletions *(Continued)*				
624-8	–Exons 2–8	28–609	In-frame deletion	
621-4	Large deletion			
621-7	Large deletion			
621-10	Large deletion			
622-2	Large deletion			
622-4	Large deletion			
622-5	Large deletion			
622-7	Large deletion			
622-12	Large deletion			
622-15	Large deletion			
622-16	Large deletion			
623-3	Large deletion			
623-4	Large deletion			
624-2	Large deletion			
624-7	Large deletion			
624-12	Large deletion			
625-1	Large deletion			
625-6	Large deletion			
625-7	Large deletion			
625-14	Large deletion			
Complex Mutations				
623-10	AT →CC	584–585	Tyr →Ala	GAC TAT AAT

[a] The mutation in the nontranscribed strand is reported.

[b] Numbering of the hprt gene according to Melton et al. 1984.

DISCUSSION AND CONCLUSIONS

MODEL FOR ESTIMATING THE MUTAGENIC POTENCY OF DNA-DAMAGING AGENTS IN T CELLS OF MICE AND RATS

The approach used in the current project to estimate the mutagenic potency of BD, BDO, and BDO_2 in splenic T cells from exposed rodents is based on a model with two components, one biological and the other mathematical. The biological component is based on the hypothesis that the time dependence of the frequency of *hprt* mutant T cells in spleen of rodents is largely due to the renewal of the peripheral T cell population; that is, that the mutations are induced primarily in T cells in bone marrow and thymus but are expressed as mutant cells that migrate to the spleen and other peripheral tissues (Jones et al. 1987b; Walker and Skopek 1993b). This hypothesis is supported by the initial detection of higher MFs in the thymus than in the spleen of (a) mice given single injections of ethylnitrosourea (Jones et al. 1987b; Walker and Skopek 1993b), (b) rats administered acrylonitrile in drinking water for 4 weeks (Walker and Walker 1997), and (c) mice and rats exposed to BD for 2 weeks (present report). Furthermore, studies in ethylnitrosourea-treated mice of various ages have demonstrated that the rate of appearance of ethylnitrosourea-induced *hprt* mutant T cells in spleen is age dependent (Jones et al. 1987b; Walker and Skopek 1993b), with increasing age apparently leading to both a reduction in the rate of renewal of peripheral lymphocyte populations (Hirokawa and Makinodan 1975) and to a decline in the number of cells migrating from the thymus to the periphery (Sollay et al. 1980). Therefore, mice and rats of nearly identical age were used in the present experiments.

In this model, clonal amplification occurs and is necessary for the transposition of mutant T cells from the thymus to the spleen, from which cells are recovered for MF analyses. The mutant T cell manifestation time for spleen (or more specifically, the time required to achieve maximum MFs in peripheral lymphoid tissues) is mainly due to this migration and, because the vast majority (95%) of thymocytes die within the thymus (Sollay et al. 1980), it

is probable that most mutations are counted only once in T cells isolated from mouse or rat spleen. However, an alternative hypothesis suggests that only a few mutations occur, perhaps in pluripotent cells of the bone marrow as prethymic events or in germinal centers of lymph nodes as postthymic events, and that the clonal amplification of these few mutations gives rise to the majority of the mutant T cells detected in spleen.

The important feature of the second hypothesis is that MFs increase due to double, triple, or higher, counting of sibling mutants. If this second premise were correct, then the mathematical approach used in the current report would be inappropriate. Nonetheless, the validity of the first hypothesis—that mutations arise primarily in prethymocytes or thymocytes in most mice and rats—is supported by (a) the shapes of the mutant manifestation curves for *hprt* MFs in spleen of BD-exposed rodents, (b) the results of earlier studies on age-related differences in the effects of elapsed time on *hprt* MFs in thymic and splenic T cells of ethylnitrosourea-treated mice (Jones et al. 1987b; Walker and Skopek 1993), and (c) the molecular analysis of *hprt* cDNA and demonstrated occurrence of few duplicate mutations among the mutant T cell clones recovered from spleen of control and BD-exposed mice (see the following discussion of data).

The roles that various compartments of the lymphoid system play in the expression and subsequent amplification of *hprt* mutant T cells are of great interest to those using *hprt* MF as a measure of exposure response to DNA-damaging agents in rodents and humans (Jones et al. 1987a,b). The degree to which mutant T cell siblings may contribute to MFs found in vivo in peripheral blood can be evaluated by assessing *hprt* mutant clones for rearrangements of the T cell–receptor gene. Interpreting such assessments is relatively straightforward in human adults because thymic activity disappears during the second decade of life. However, comparison of data on *hprt* mutations and T cell–receptor gene rearrangements in rodents is complicated by the maintenance of thymic activity throughout the life span. Therefore, one can never determine with certainty if duplicate *hprt* mutations in T cell clones of a given animal are prethymic, thymic, or postthymic in origin.

The occurrence of a substantial number of *hprt* mutant T cell clones (isolated from spleen) with the same mutation in a given mouse or rat would suggest that these mutations originated from clonal expansion of a mutant pluripotent cell or memory cell. Indeed, some of the outliers in MFs observed in the present project may be explained by (a) prethymic expansions of mutations in pluripotent bone marrow cells leading to increased recovery of the same mutation in T cell clones with different T cell–receptor gene arrangements, or (b) postthymic clonal expansions resulting in increased recovery of the same mutation in T cell clones with the same T cell–receptor gene arrangements.

Nonetheless, the *hprt* MF data reported here demonstrate that such clonal expansions occur at a low frequency in control and BD-exposed rodents and have little impact upon the overall shape of the mutant manifestation curve for *hprt* MFs in T cells recovered from mouse and rat spleen. On the other hand, an occasional duplicate mutation among a group of *hprt* mutant clones isolated from the spleen of a single animal is more likely to originate from prethymocytes or thymocytes, and it may represent either mutant siblings or independent mutations. Thus, *hprt* MFs measured in the peripheral lymphocytes of rodents reflect the varied dynamics of mutation fixation and T cell kinetics and, as such, MFs should not be corrected for duplicate mutations to derive a "mutation frequency" of uncertain value. Rather, curve fitting (and then integrating a polynomial model with respect to time) appears particularly useful for handling *hprt* MF data for rodent T cells because it pools random variability that may be biologically based around fitted curves, and, therefore, provides more residual degrees of freedom, better precision, and more statistical power for comparing parameters across experimental groups (Health Review Committee's Commentary in Beland 1995).

Results of the molecular analysis of *hprt* cDNA from BD-exposed mice (Specific Aim 4) revealed that the majority of mutant splenic T cell clones contained independent mutations and that the few duplicate mutations observed had an insignificant impact upon MFs in each animal if the duplicates were considered to be mutant siblings. Correction of duplicate mutations to determine a "mutation frequency" for each animal (Table 13) resulted in a mean [± SD] "mutation frequency" of 9.8 [± 2.9] × 10^{-6} compared with an MF of 10.7 [± 3.1] × 10^{-6} when duplicate mutations remained uncorrected. The 8% reduction in mutagenic response provided by the mean "mutation frequency" value in this group of BD-exposed mice represents only 27% of the total variance (shown by SD) associated with the mean MF for the group. Thus, the p values (0.002) are the same whether mean "mutation frequencies" or separate MFs of control and exposed groups are compared. These data demonstrate that the use of MFs is warranted in estimating the mutagenic response in T cells of rodents and that the systematic molecular analysis of hundreds or thousands of *hprt* mutant clones from all animals in an experiment to derive "mutation frequencies" provides no advantage.

The mathematical approach used here to derive an estimate of total mutagenic burden for interspecies comparison

is novel: It integrates the areas under the mutant T cell manifestation curves. This integration is approximately equal to multiplying the ratio of the number of *hprt* mutant cells per number of wild-type cells by time ([number of mutants/number of wild-type cells] × time). This latter term (enclosed in parentheses) by itself is not very meaningful; however, when multiplied by the average number of wild-type cells passing through the spleen per unit of time (number of wild-type cells/time), the result represents the number of mutants over time. Therefore, for the area under the curve to be a meaningful value for comparing the mutational burden in mice and rats, two assumptions must be made: (a) the rate at which cells pass through the spleen should be similar in the two species, and (b) the same fraction of all mutant T cells produced in the body should pass through the spleen. No systematic studies have evaluated these two assumptions in mice and rats; however, limited experimental data permitting comparisons of T cell kinetics and mutagenic response in splenic T cells from these two rodent species (Sollay et al. 1980; Jones et al. 1987b; Crippen and Jones 1989; Aidoo et al. 1991;Walker and Skopek 1993b) suggest that these criteria are likely to be valid under the experimental conditions reported here. Moreover, the data in Figure 5 demonstrate that the alternate approach of calculating simple ratios between MFs at different times after exposure of mice and rats has limited value.

The success of the rather complicated mutagenic potency computation outlined in the Methods and Study Design section depends on a good estimate of the change in MF over time after last exposure. The robust regression method employed to estimate polynomials for these profiles down-weights outliers presumably caused by clonal expansion. Nonrobust estimates of polynomials are apt to be excessively influenced by such expansions and thereby yield excessively large integrated potency (area) estimates, which in turn lead to overestimated mutagenic potencies. Polynomial models are often most appropriate for short time periods because, rather than approaching asymptotes, they tend to diverge outside the biologically meaningful range at longer time intervals.

BACKGROUND MUTANT FREQUENCIES IN SPLENIC T CELLS OF MICE AND RATS

The variations in *hprt* MFs in control mice and rats among individual groups of animals in the same experiment or between experiments in this project were somewhat smaller than the variations obtained by other investigators (Jones et al. 1987b; Aidoo et al. 1991; Skopek et al. 1992; Cochrane and Skopek 1994; Walker and Skopek 1993; Tates et al. 1994, 1998; Walker et al. 1996, 1997, 1999). Many factors could have contributed to this observation. First, mice and rats of the same strains and ages were used throughout several sets of experiments. Second, the same researchers conducted all experiments, thus avoiding the use of different criteria in counting mutant colonies. Third, more control animals were used in this project than in some others (in Specific Aim 2, for example, the number of control animals included 40 mice and 36 rats for BD, 26 mice and 24 rats for BDO, and 27 of each species for BDO_2).

MUTAGENIC EFFECTS OF BD EXPOSURE IN MICE AND RATS

As noted earlier, the mutagenicity of BD was previously demonstrated at both the *hprt* and *lacI* loci of conventional and transgenic mice, respectively. BD caused increased *hprt* MFs in splenic T cells of preweanling $B6C3F_1$ mice (Cochrane and Skopek 1994b) and several strains of adult mice (Tates et al. 1994, 1998). The mutagenic effects of BD were also detected in the *lacI* transgene of $B6C3F_1$ Big Blue™ mice (Sisk et al. 1994; Recio et al. 1996b).

The initial experiments performed in this project provided the first definitive evidence that BD is an in vivo mutagen in the rat as well as in the mouse. For example, in the first exposure-duration experiment, *hprt* mutations accumulated in T cells over time during exposures of both mice and rats to 1,250 ppm BD for 2 weeks (Figure 1). Nonetheless, the increases in MFs over background were easier to recognize in mice than in rats due to two factors. First, the magnitude of BD-induced effects was much greater in mice than in rats. Second, mouse necropsies were performed at the optimal time for detecting MFs, whereas rat necropsies were performed at a suboptimal time. All animals in this exposure-duration experiment evaluating MFs in thymic T cells were killed at 2 weeks after the last exposure; however, the experiment assessing phenotypic expression of *hprt* mutations in thymic T cells (Figure 2) shows that maximum MFs were achieved at 2 weeks after exposure in mice, but at 3 weeks after exposure in rats. These data indicate that the accumulation of BD-induced mutations in rat thymus after 1 week rather than 2 weeks of exposure (Figure 1) would have been demonstrated better if the rats had been necropsied at 3 weeks after exposure to coincide with the phenotypic expression that yields maximum MFs in rat thymic T cells (Figure 2).

The follow-up exposure-duration experiment demonstrated that *hprt* mutations continued to accumulate in T cells beyond 2 weeks of exposure. (Mice and rats were exposed to 625 ppm BD for 4 weeks, Figure 6.) The rate of accumulation of mutations over this exposure period was greater in mice than in rats, indicating that, with the

extension of BD exposure time, the species differences in mutagenic response between mice and rats should continue to diverge. However, it is not known for how long beyond 4 weeks of exposure mutations would continue to accumulate before reaching a plateau.

In contrast to these results, no significant difference in the accumulation of mutations was observed in the *lacI* gene of bone marrow from transgenic mice exposed to 625 ppm BD for 1 week or 4 weeks (Recio et al. 1996a). The discrepancy between the exposure-duration study in *lacI* transgenic mice and the present experiments may be related to several factors, including (a) different tissues and cell types were studied, (b) MFs were determined at different times after exposure, and (c) MFs were measured at one time point after exposure in the *lacI* mutation experiment rather than multiple time points as in the current *hprt* mutation experiment.

The dose-response curves for *hprt* mutant splenic T cells were nonlinear in both mice and rats. In mice, the increases in MFs were dose dependent, and the unit dose of BD had more effect at lower exposure concentrations than at higher concentrations (Figure 8). This pattern of response may be related to several factors, including reduced efficiency of BD bioactivation, increased rate of detoxification of BD metabolites, and lower efficiency of mutation induction by BD metabolites as the BD exposure is increased. In transgenic mice exposed to BD concentrations of 0, 62.5, 625, or 1,250 ppm for 4 weeks, a plateau occurred in the *lacI* MFs between 625 and 1,250 ppm BD. However, when the *lacI* MFs were adjusted by the independence of mutant cells, the shape of the dose-response curve (Recio et al. 1996b) became similar to that found in the present report. In rats, the increases in MFs were also dose dependent (Figure 8), but the increases of MFs were only significant at 625 and 1,250 ppm BD.

MUTAGENIC EFFECTS OF BDO EXPOSURE IN MICE AND RATS

Two research groups have previously reported that exposure of mice to the racemic mixture of BDO leads to increased *hprt* MFs in splenic T cells. Cochrane and Skopek (1994b) found 3.4- to 7.2-fold increases in *hprt* MFs in splenic T cells of preweanling mice necropsied 2 weeks after a series of three intraperitoneal injections of 60, 80, or 100 mg BDO/kg every other day over 6 days. Tates and colleagues (1998) detected a 3.8-fold increase in *hprt* MFs in splenic T cells of adult mice necropsied 39 days after delivery of three intraperitoneal doses of 100 mg/kg BDO at 48-hour intervals between doses. In the present report, inhalation exposures to BDO caused significantly increased *hprt* MFs in mice only at 7 weeks after exposure, and this response could have been missed if MFs had been measured at only one time point instead of looking at the change in MFs over time (Figure 9). Nevertheless, at 7 weeks after exposure, the dose response for *hprt* MFs in BDO-exposed mice was supralinear, indicating a higher mutagenic efficiency at the lower exposure concentration (2.5 ppm) than at the higher exposure concentration (25 ppm) (Figure 10). The mutagenic effects of racemic BDO were also evaluated at the *lacI* transgene following inhalation exposure of $B6C3F_1$ transgenic mice to BDO (25 ppm, 6 hours/day, 5 days/week, for 2 weeks), but no increases in *lacI* MFs were observed in the spleen and bone marrow of exposed mice or controls (Recio et al. 1998). Induced mutations at the *hprt* locus, but not at the *lacI* locus, in splenic lymphocytes were also found in mice treated with cyclophosphamide or ethylene oxide (Sisk et al. 1997; Walker et al. 1997, 1999). Possible reasons for the varying sensitivity of the *hprt* mutation assay and *lacI* mutation assay for detecting chemically induced mutations have been discussed elsewhere (Skopek 1995; Skopek et al. 1995; Walker et al. 1996, 1999).

BDO showed no mutagenic response in conventional rats at *hprt* (this report), but a significant response was found at *lacI* in transgenic rats (Saranko et al. 1998). The *hprt* MFs were not increased in rats exposed to BDO by inhalation (this study, Figure 9) or by intraperitoneal injections (Tates et al. 1998). However, a trend toward increased MFs was observed at 7 weeks after the last exposure in the current study, and this increase might have been significant if more BDO-exposed rats had been necropsied at that time point. In the study by Tates and colleagues (1998), the acute toxicity of BDO in rats prevented the planned treatment with three injections of 100 mg/kg, and this limitation may have reduced the chance to detect a mutagenic response in rats using a treatment regimen that induced a positive mutagenic response in mice. In contrast, F344 *lacI* transgenic rats had elevated MFs in bone marrow, but not in spleen, after 2 weeks of inhalation exposure to 25 ppm BDO (Saranko et al. 1998).

MUTAGENIC EFFECTS OF BDO_2 EXPOSURE IN MICE AND RATS

Several research groups have previously evaluated the mutagenicity of (±)-BDO_2 in mice and rats. Cochrane and Skopek (1994b) reported 3.8- to 10.8-fold increases in *hprt* MFs in splenic T cells of preweanling mice necropsied 2 weeks after intraperitoneal injections every other day with 7 (3 doses), 14 (2 doses), or 21 (2 doses) mg BDO_2/kg. In contrast, no significant increase in *hprt* MFs was found in splenic T cells of adult mice and rats exposed to BDO_2 either by intraperitoneal injection or via ingested drinking water (Tates et al. 1998). Finally, inhalation exposures of $B6C3F_1$ *lacI* transgenic mice and F344 *lacI* transgenic rats

to 5.0 ppm BDO_2 for 2 weeks (6 hours/day, 5 days/week) did not increase *lacI* MFs in bone marrow and spleen (Recio et al. 1998). Reasons used to explain the lack of a detectable mutagenic response to other compounds at the *lacI* locus compared with a positive response at the *hprt* locus might be applicable here. First, large deletions induced by BDO_2 (Cochrane and Skopek 1994a; Steen et al. 1997a) and other clastogenic agents are poorly recovered using the *lacI* transgene mutation assay. Secondly, the frequency of background mutations at the *lacI* locus is relatively high (Skopek et al. 1995; Walker et al. 1996, 1999).

In the present study, (±)-BDO_2 exposures caused significantly increased *hprt* MFs in splenic T cells of both rats and mice following 4 weeks of inhalation exposure and, interestingly, the mutagenic effects were greater in rats than in mice (Figure 11). This greater mutagenic response to (±)-BDO_2 exposures in rats may be related to at least two factors. First, the rate of hepatic clearance of BDO_2 by hydrolysis and glutathione conjugation in rats is 2.3-fold lower than in mice (Boogaard and Bond 1996; Boogaard et al. 1996), which could lead to higher blood and tissue levels of BDO_2 in rats than in mice exposed exogenously to the same concentration of BDO_2. Second, rat lymphocytes appear to tolerate the cytotoxic effects of BDO_2 better than mouse lymphocytes (Henderson et al. 1997), and greater T cell survival would favor higher MFs in the rat.

The results of various studies of *hprt* MFs in splenic T cells of BD-, (±)-BDO-, and (±)-BDO_2-exposed mice and rats (Cochrane and Skopek 1994b; Tates et al. 1994, 1998; Meng et al. 1998a; this report Specific Aims 1 and 2) are difficult to compare quantitatively because of differences in (a) the ages and strains of the animals used, (b) the routes of exposure, (c) the exposure regimens, (d) the time allowed for mutant manifestation, and (e) the number of sampling times after exposure. Nevertheless, the *hprt* MF data discussed above indicate that the mutagenic effects of exposures to BD, BDO, and BDO_2 can be detected more effectively in young than in adult mice and rats. Tates and colleagues (1998) suggested that these age-specific differences in mutagenic response may be primarily related to age-dependent differences in biotransformation of BD, to thymic activity, and to trafficking of T cells. These issues will be considered further in a future paper concerned with the relations among chemical exposures, age of animals at treatment, T cell toxicity, T cell kinetics, and T cell mutagenicity (Walker and Skopek 1993b; and unpublished data).

COMPARISON OF THE MUTAGENIC POTENCIES OF BD, BDO, AND BDO_2 EXPOSURES IN MICE AND RATS

Following the initial mutagenicity experiments reported here (Specific Aim 1), the species differences in the mutagenic potency of BD in T cells of female $B6C3F_1$ mice and F344 rats appeared to be similar in magnitude to the species differences in BD metabolism; however, neither species differences in metabolism nor mutagenic potency correlate well quantitatively with the species differences in tumor susceptibility. As noted earlier, BD is a potent carcinogen in mice and a weak carcinogen in rats. For example, increases in the incidence of lung tumors were observed in $B6C3F_1$ mice at exposure concentrations as low as 6.25 ppm BD (Melnick et al. 1990), whereas only minor increases in the incidence of mammary tumors were found in Sprague-Dawley rats exposed to 1,000 ppm BD (Owen et al. 1987) under bioassay conditions.

Species differences in the rates of metabolism became of paramount interest when these carcinogenic responses in mice and rats were reported (Birnbaum and Keller 1996), and significant species differences in the ratios of the mutagenic metabolites, BDO and BDO_2, were subsequently found in male mice and rats exposed to the same amounts of BD (Henderson et al. 1996; Himmelstein et al. 1994, 1995, 1996; Thornton-Manning et al. 1995a,b, 1996). For instance, following exposure to 62.5 ppm BD for 4 hours, male $B6C3F_1$ mice had 8- and 40-fold higher blood concentrations of BDO and BDO_2, respectively, than were found in male Sprague-Dawley rats (Thornton-Manning et al. 1995a, 1996). Similar differences were found in other tissues, including thymus. However, in a gender comparison of BD metabolism in Sprague-Dawley rats (62.5 ppm for 6 hours), tissue levels of BDO were similar in both sexes whereas levels of BDO_2 were 3- to 7-fold higher in females than in males (Thornton-Manning et al. 1995b, 1996).

These species differences in the ratios of BDO and BDO_2 are roughly comparable to the species difference in the mutagenic potency of BD in female $B6C3F_1$ mice versus F344 rats. However, fruitful comparisons of the metabolite ratios and mutagenic potency ratio are complicated by at least two factors. First, the levels of BDO and BDO_2 in various tissues have not been determined in BD-exposed female mice. Given the high susceptibility of female mice to BD-induced cancers, it is important to conduct a gender comparison of BD metabolism in $B6C3F_1$ mice as a means of defining any sex differences in BDO and BDO_2 formation in female and male mice, as well as in female mice versus male and female rats. In addition, the overall MF data obtained in the initial experiments (Figures 1–3) suggest that the species difference in *hprt* MFs would continue to diverge with further exposure to BD, with the induced *hprt* MFs (and mutagenic potencies) becoming increasingly greater for some unknown period of time beyond a 2-week exposure in BD-exposed mice compared with exposed rats.

These issues were addressed further in studies of *hprt* MFs in mice and rats exposed by inhalation to a range of BD concentrations for 2 or 4 weeks (Specific Aim 2). Therefore, it is notable that the estimated mutagenic potencies in splenic T cells for mice and rats exposed to 625 ppm BD for 4 weeks (Table 4) were similar to those observed for mice and rats exposed to 1,250 ppm BD for 2 weeks (Table 1), suggesting that both exposure concentration and exposure duration are important in determining the magnitude of mutagenic response to this chemical. Moreover, the mutagenic potency studies overall (Specific Aims 1 and 2) demonstrated that defining the change in MFs over time, compared with measuring MFs at one time point, is advantageous because the ratio of MF measurements in mice and rats at any single time point after exposure tends to overestimate or underestimate the species difference in mutagenic response to BD.

The potential contribution of racemic mixtures of BDO and BDO_2 to the mutagenicity of BD in mice can be estimated to a reasonable extent by comparing the mutagenic potencies of BD, BDO, and BDO_2 at comparable internal doses (Table 4). However, the use of only racemic mixtures of BDO and BDO_2 for inhalation exposures limits the value of these experiments for comparison with metabolically produced epoxides during BD exposure of rodents. When comparable internal doses of epoxides are achieved, the effects of (±)-BDO and (±)-BDO_2 are really being compared with corresponding blood levels of the varied stereochemical configurations of BDO and BDO_2, respectively, that result from in situ metabolism in BD-exposed mice and rats. Thus, the actual stereochemical forms of the compounds used and the expected metabolites are different across the inhalation studies, and these differences could be significant in terms of toxicity and mutagenicity in T cells.

On the other hand, the inhalation studies reported here reveal important trends in mutagenic response that begin to distinguish the relative contribution of epoxy metabolites and specific stereochemical configurations to the induction of mutations in BD-exposed mice and rats. For example, the mutagenic potency of 2.5 ppm (±)-BDO, a dose that appears to give a similar blood level of BDO as found in mice exposed to 62.5 ppm BD (discussed above), had only one third of the mutagenic potency of a direct 62.5 ppm BD exposure in mice, indicating that at least (±)-BDO is not a major contributor to the mutagenicity of BD in mice at exposure concentrations of ≤ 62.5 ppm of the parent chemical (Table 4). Instead, the mutagenic potency of 62.5 ppm BD in mice may be explained largely by its ultimate conversion to BDO_2 (Table 4). Furthermore, because BDO can be converted to BDO_2 in vivo, the observed mutagenic response in BDO-exposed mice could also come from BDO_2 derived from BDO. This possibility is supported by the fact that the dose-response curve for *hprt* MFs in BDO-exposed mice was supralinear (approaching a plateau at the higher exposure concentrations), whereas the dose-response curve for BDO-derived DNA adducts was linear in mice exposed to BD at concentrations ranging from 20 to 625 ppm (Koc et al. 1999). Conversely, the T cell toxicity data and dose-response curves for *hprt* MFs in (±)-BDO_2-exposed mice suggest that other epoxy metabolites in various stereochemical configurations are likely to be responsible for the incremental mutagenic effects at higher exposures to BD.

Figure 18 provides a useful comparison of the dose-response curve for mutagenic potency of BD in splenic T cells of female $B6C3F_1$ mice exposed for 4 weeks with the dose-response curves for induced tumors (that is, tumor incidence in exposed mice minus tumor incidence in control mice) of two types, lung neoplasms and lymphocytic lymphomas, in female $B6C3F_1$ mice exposed to BD for up to two years (Melnick et al. 1992). The induced lung tumor incidences per unit dose are 3.84, 1.80, 0.85, 0.37, and 0.12 (%/ppm) for BD exposure concentrations of 6.25, 20, 62.5, 200, and 625 ppm, respectively (Melnick et al. 1990). They are consistent with the findings in the current

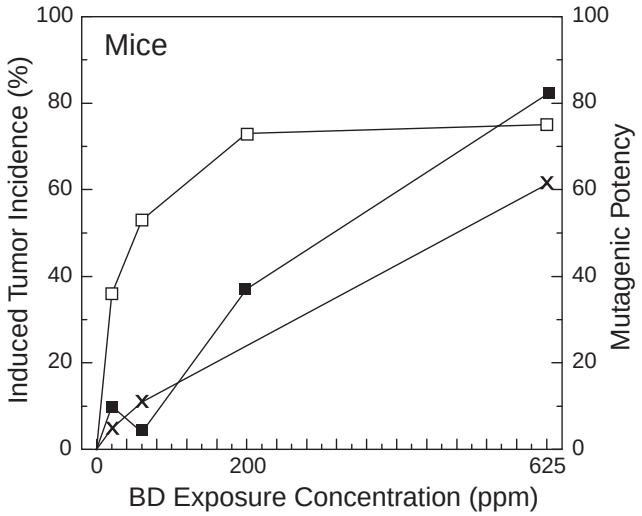

Figure 18. Comparison of the effects of BD exposure concentration on mutagenic potency at the *hprt* locus of T lymphocytes and the incidence of induced cancers in exposed female mice. In the mutagenicity experiments from the present study, groups of female $B6C3F_1$ mice (4–5 weeks old, n = 6–9 per group) were exposed to 0, 20, 62.5, or 625 ppm BD for 4 weeks (6 hours/day, 5 days/week), and MFs in splenic T cells were measured at 4 weeks after the last exposure. In the carcinogenesis studies (Melnick et al. 1992), female $B6C3F_1$ mice (6.5 weeks old, n = 70–90 per group) were exposed to 0, 20, 62.5, 200, or 625 ppm BD for up to 2 years (6 hours/day, 5 days/week). The incidence of BD-induced tumors at a given exposure concentration = [number of BD-exposed mice with a tumor of a specific type] – [number of control mice with the same tumor type]. Mutagenic potency at *hprt* (×), incidence of lung cancer (□), incidence of lymphocytic leukemia or lymphoma (■), with tumor incidence data from Table 6 in Melnick et al. 1992.

hprt mutagenicity study in that the unit dose of BD has a greater effect at lower exposure concentrations than at higher concentrations. The implication of these MF and tumor incidence data is that the mutagenic metabolite (or metabolites) acting at lower exposure concentrations is driving the carcinogenic response in the lungs of BD-exposed mice. Henderson and colleagues (1998) recently addressed the question of whether (±)-BDO_2 might be the ultimate carcinogen accounting for the high sensitivity of mice to BD-induced lung tumors. The carcinogenic potential of (±)-BDO_2 was evaluated in female $B6C3F_1$ mice and Sprague-Dawley rats exposed by inhalation to 0, 2.5, or 5 ppm for 6 weeks (6 hours/day, 5 days/week); the only significant neoplastic response in animals necropsied up to 18 months after exposure was the occurrence of squamous cell carcinomas in the upper respiratory tract of (±)-BDO_2-exposed rats. These results do not eliminate the possibility that (±)-BDO_2 or *meso*-BDO_2 contributes to the origin of BD-induced lung tumors in mice, but they do demonstrate that much work is still needed to delineate the metabolites, stereochemistry, and mutagenic mechanisms involved. Conversely, the differing shapes of the dose-response curves for *hprt* MFs in splenic lymphocytes and for the incidence of induced lymphomas in BD-exposed mice suggest that mutagenic metabolites acting at higher BD exposures are primarily responsible for the generation of lymphoma. Melnick and colleagues (1990) have observed that, at comparable total exposures, the incidence of lymphoma was greater with exposure to a higher concentration for a short time than with exposure to a lower concentration for an extended duration. Thus, the *hprt* MF and tumor incidence data together suggest that either different DNA-reactive metabolites or different balances in the amounts of mutagenic metabolites are responsible for the tissue-specific differences in the carcinogenicity of BD in mice.

Although exogenous exposure to (±)-BDO_2 was more mutagenic in the rat than in the mouse (Figure 11), the body of evidence comparing species differences in metabolism of BD (see Himmelstein et al. 1997) indicates that the internal doses of (±)-BDO_2 and *meso*-BDO_2 to various tissues is too low to have major mutagenic consequences when rats are exposed to BD. For example, the blood and tissue levels of BDO_2 were 40- to 160-fold lower in rats than in mice exposed to 62.5 ppm BD (Thornton-Manning et al. 1995a), and this species difference may explain the lack of mutagenic response to 62.5 ppm BD in rats compared with a positive response in similarly exposed mice (Figure 8). Furthermore, blood levels of BDO_2 did not increase in rats exposed to 625, 1,250, or 8,000 ppm BD (Thornton-Manning et al. 1998), but the level of BDO was 14-fold higher in rats exposed to 625 ppm BD than in rats exposed to 62.5 ppm BD (Himmelstein et al. 1994). Thus, the mutagenic activity of metabolites other than BDO_2 appears to be responsible for the lower carcinogenic potency of BD in rats compared with mice.

Although much has been learned from experiments examining the mutagenic potential of racemic mixtures of BDO and BDO_2, potential contributions of other BD metabolites and other stereochemical configurations need to be evaluated. For example, the relative amounts of (±)-BDO_2 and *meso*-BDO_2 formed in vivo after BD inhalation have not been determined in rodents; recent DNA adduct studies of livers from BD-exposed mice and rats have demonstrated the accumulation of adducts at the N7 position of guanine presumably derived from both (±)-BDO_2 and *meso*-BDO_2 (Oe et al. 1999). The *meso*-BDO_2 adducts were found to persist longer in mouse liver than in the rat liver, and it is conceivable that *meso*-BDO_2 may be more carcinogenic to the mouse than (±)-BDO_2. Therefore, future studies should be aimed at determining the relative amounts of (±)-BDO_2 and *meso*-BDO_2 in various tissues in vivo and assessing their relative mutagenic potency following inhalation exposures of mice and rats to the diastereomeric forms of BDO_2 versus BD.

Accumulating data suggest a potential role for BDO-diol in BD-induced mutagenicity, especially at higher exposure levels of BD. The relative amounts of BDO-diol formed in vivo, compared with amounts of BDO and BDO_2, have not been determined in rodents, but large amounts of BDO-diol are assumed to be formed in animals on the basis of in vitro metabolism studies of BD. DNA adducts that can be formed from both BDO_2 and BDO-diol have been recently found to occur at much higher concentrations than BDO-derived DNA adducts in BD-exposed mice. Furthermore, Koc and colleagues (1999) present evidence that the majority of the adducts that could arise from BDO_2 and BDO-diol are formed by the latter metabolite. Therefore, it will be important to determine the relative amounts of BDO-diol in various tissues in vivo and to evaluate the relative mutagenic potency of BDO-diol following inhalation exposures of mice and rats to either BDO-diol or BD. Ongoing and future comparisons between studies of mutations and DNA adducts should greatly aid in the ultimate identification of the roles and stereochemistry of BD metabolites present after mice and rats are exposed to low and high concentrations of BD.

COMPARISON OF MUTATIONAL SPECTRA IN THE *hprt* GENE OF T CELLS FROM MICE AND RATS EXPOSED TO BD OR BDO_2

The mouse and rat lymphocyte *hprt* mutation assays have been shown to be valuable for investigating the mutagenic

potency and specificity of experimental alkylating agents and potential environmental carcinogens in vivo, but the optimization of these models has been hindered by the relatively poor growth potential of rodent T lymphocytes in vitro (Jones et al. 1985a,b; Aidoo et al. 1991, 1993, 1994, 1997; Skopek et al. 1992, 1996; Tan et al. 1993; Walker and Skopek 1993; Cochrane and Skopek 1994b; Tates et al. 1994, 1998; Mittelstaedt et al. 1995; Walker et al. 1996, 1997, 1998; Chen et al. 1998; Meng et al. 1998a). This difficulty has seriously impeded the molecular analysis of *hprt* mutant lymphocytes isolated from both mice and rats (Skopek et al. 1992; Walker and Skopek 1993; Mittelstaedt et al. 1995); only two reports employed limited expansion of mouse T cell clones for mutation analysis via Southern blotting of genomic DNA (Jones et al. 1987a) or RT-PCR of *hprt* mRNA (Tan et al. 1993). Instead, DGGE has been used more extensively as a temporary expedient for the molecular analysis of T cell mutations in rodents because DGGE provides an analytical means of purifying mutant sequences from contaminating wild-type sequences with the goal of DNA sequencing of individual *hprt* exons (Skopek et al. 1992; Walker and Skopek 1993; Mittelstaedt et al. 1995). The disadvantages of the DGGE sequencing approach are twofold. It is labor intensive, and it can only detect base-pair substitutions and small insertions or deletions (Walker et al. 1996, 1997; Chen et al. 1998). Thus, the recent development of methods for substantial expansion of mutant T cell clones from mice and rats allows the use of various methods to identify a broader range of DNA lesions over the entire *hprt* gene of unexposed and exposed animals (Chen et al. 1998; Meng et al. 1998b).

A major advantage of inspecting expanded *hprt* mutant T cell clones for mutations in control and BD-exposed mice is readily apparent when the summary of mutations identified by RT-PCR analysis of *hprt* mRNA (Table 11) is compared with the summary of mutations identified by the DGGE approach (Table 5). The most striking feature in the RT-PCR analysis of mutations in BD-exposed mice is the occurrence of a significant increase in large deletions, ones that encompass the whole *hprt* gene. This result is in keeping with earlier in vitro experiments in BDO- and BDO_2-exposed human lymphoblastoid cells (Cochrane and Skopek 1994a; Steen et al. 1997a,b), suggesting that exposure of mice to BD should lead to large deletions as well as point mutations. Furthermore, the finding of 51% single-exon deletions and large deletions in *hprt* cDNA of BD-exposed mice reveals the limited value of in vivo mutational spectra data obtained with approaches that basically detect only point mutations, such as DGGE analysis of *hprt* exon 3 (present study) and analysis of the *lacI* gene (Recio et al. 1995, 1996a,b) in similarly exposed rodents. In fact, although the DGGE analysis of mutant clones from BD- and (±)-BDO_2-exposed mice and rats represents a monumental amount of work, the resulting mutational spectra data did not produce very definitive results. Nevertheless, a few salient observations need to be made concerning DGGE-identified point mutations in rodent studies involving BD, BDO_2, and several other direct or indirect alkylating agents.

The proportions of *hprt* exon 3 mutations by DGGE analysis of mutant clones from BD- and (±)-BDO_2-exposed $B6C3F_1$ mice were lower than those reported for $B6C3F_1$ mice treated with cyclophosphamide (22%) (Walker et al. 1999) or ethylene oxide (18%) (Walker and Skopek 1993a; Walker et al. unpublished data). These observed differences may be due largely to the fact that deletions involving the loss of one or more exons or the loss of the entire *hprt* gene will go undetected using DGGE analysis. Because BDO_2 is a bifunctional alkylating agent that has been shown to induce large deletions and DNA rearrangements as well as point mutations in vitro (Cochrane and Skopek 1994a, 1997a; Steen et al. 1997a), the lower fraction of mutations in exon 3 of BD- and BDO_2-exposed mice may be attributable in part to the relatively increased occurrence of deletions and rearrangements that do not contribute observable mutations in exon 3. This conclusion is supported by the detection of an increased frequency of large deletions in *hprt* cDNA of expanded mutant clones from BD-exposed mice compared with the frequency in controls (Table 11).

The spectrum of mutations observed in *hprt* exon 3 of T cells from BD-exposed $B6C3F_1$ mice was significantly different from the limited sequence data from control $B6C3F_1$ mice and from robust spectra data from $B6C3F_1$ mice treated with cyclophosphamide (Walker et al. 1999), ethylnitrosourea (Skopek et al. 1992; Walker et al. 1996), or ethylene oxide (Walker and Skopek 1993a). The limited *hprt* mutational spectra data from earlier studies of BD (13 exon 3 mutations) and ethylene oxide (18 exon 3 mutations) in mice (Walker and Skopek 1993a; Cochrane and Skopek 1994b) suggested that the types and locations of mutations produced by these agents might overlap substantially, but the similarities are less striking when the *hprt* mutation data for BD in the current study (56 exon 3 mutations) are compared with an expanded data set for mice exposed to ethylene oxide by intraperitoneal injections or inhalation (57 and 48 exon 3 mutations, respectively) (Walker and Skopek 1993a; Walker et al. unpublished data) (Table 12). A more detailed comparison of the mutagenic specificities of BD and ethylene oxide in mice will be presented in a report on the mutagenicity of ethylene and ethylene oxide in mice and rats.

In contrast, a high degree of overlap was noted in the mutational spectra data between BD-exposed mice and rats, between BD- and (±)-BDO_2-exposed mice, and between BD- and (±)-BDO_2-exposed rats in terms of the sites with base substitutions, the mutations found at those sites, and the relative occurrence of the most frequently observed base substitutions. For example, half of the mutation sites with base substitutions in BD-exposed mice (12/21 sites) and rats (12/24) were common to both species, and 75% of these shared sites had identical mutations (Tables 7 and 8). The same degree of shared sites for base substitutions and identical mutations were found in BD- and BDO_2-exposed mice (Tables 7 and 9). Furthermore, a large number of the exon 3 mutations (22% to 38%) observed in BD-exposed mice and rats and in BDO_2-exposed mice occurred in a particular run of six guanines (bp 207–212). These data alone suggest a high probability that some mutations were induced through similar mutagenic mechanisms in these BD-exposed mice, BD-exposed rats, and (±)-BDO_2-exposed mice.

Among the different classes of base substitutions, only A•T→C•G transversions were found to be increased in *hprt* exon 3 of BD- and BDO_2-exposed mice and rats, and the majority of these transversions (23/33) were found at bp 216 in multiple animals of all four exposure groups. Remarkably, 30 of 33 thymine bases at risk in A•T→C•G transversions in all of the BD- and BDO_2-exposed animals combined were located on the nontranscribed strand. Thus, bp 216 appears to be a highly mutable site for BD metabolites in the context of mutations produced specifically in *hprt* exon 3.

The overall occurrence of G•C→C•G transversions in *hprt* exon 3 was significantly increased in BD-exposed mice and appeared to be increased in BDO_2-exposed mice, with the majority of these base substitutions located at bp 211 or 212. Of the 26 guanines at risk in these G•C→C•G transversions, 25 were situated on the nontranscribed strand of *hprt* exon 3. This may reflect either preferential repair in the transcribed strand or differential fidelity of leading and lagging strand replication of adducted DNA (Skopek et al. 1992). On the other hand, no increase occurred in the frequency of G•C→C•G transversions in the limited set of base substitutions found in *hprt* cDNA from expanded mutant clones of BD-exposed mice when compared with the frequency in controls.

G•C→A•T transitions at bp 208 identified by DGGE analysis were the most frequently observed mutation in *hprt* exon 3 of (±)-BDO_2-exposed mice (that is, 12 occurrences in 8 mice, within a total of 66 exon 3 mutations), indicating that these mutations were likely to be related to BDO_2 exposure. This mutation also occurred a few times in BD- and BDO_2-exposed rats (4/59 and 3/51 exon 3 mutations, respectively), but it was not seen in BD-exposed mice. Yet, the occurrence of G•C→A•T transitions at bp 208 in *hprt* cDNA of expanded mutant clones from 3 of 5 BD-exposed mice, but not in clones from controls, suggests that this mutation in the mouse is produced by one or more BD metabolites.

The in vitro mutational specificities of the racemic mixtures of BDO_2 and BDO have been examined in several studies using cultured human TK6 lymphoblastoid cells to evaluate the potential role of each metabolite in the mutagenicity of BD. One major advantage of this culture system is that TK6 cells do not have the metabolic capacity for in vitro conversion of BDO_2 or BDO to other epoxy compounds. In addition, *hprt* mutant TK6 clones have the high growth potential needed for assessing the occurrence of large deletions. At the same time, the inability of TK6 cells to bioactivate BD has precluded direct comparisons between DNA damage and mutational spectra produced by exposure to the parent compound and the damage and mutational spectra produced by its DNA-reactive metabolites. Howbeit, Cochrane and Skopek (1994a) exposed TK6 cells to BDO and BDO_2 and demonstrated via Southern blot analysis that 54% of the *hprt* mutants isolated after BDO_2 exposure, as well as 12% of the mutants isolated after BDO exposure, resulted from deletion of the *hprt* sequence. The frequency of these alterations was not determined among spontaneous *hprt* mutants from unexposed cells; but comparisons of the increase in *hprt* MF in exposed cells over background MF and the frequency of deletions among *hprt* mutants in exposed cells indicated that a substantial fraction of the deletions observed after BDO_2 exposure were induced, whereas the deletions found after BDO treatment could be background in origin. Steen and colleagues (1997a,b) went a step further and determined the spectra of mutations induced at the *hprt* locus of BDO_2- and BDO-treated TK6 cells and analyzed a set of spontaneous *hprt* mutants from the same stock cultures. Compared with the spectra of background mutations, the outstanding features of the mutational spectra in exposed cells were increased frequencies of A•T→T•A transversions after BDO_2 or BDO exposure and a high frequency of genomic deletions affecting the 5′ region of *hprt* following BDO_2 exposure. All of the A•T→T•A transversions in BDO-induced mutants occurred with an adenine in the nontranscribed strand, whereas the majority of these transversions in BDO_2-induced mutants occurred with an adenine in the transcribed strand. These in vitro data indicate that BDO exposures lead primarily to base substitutions, whereas BDO_2 exposures can produce large deletions, rearrangements, or substitutions.

These two research groups have also conducted studies in BD-, (±)-BDO_2-, and (±)-BDO-exposed mice to investigate the relative contributions of these two metabolites to the mutational specificity of the parent compound (BD) in vivo. The work of Cochrane and Skopek (1994b), using the endogenous *hprt* gene as a mutational target in mouse T cells, was a point of departure for the studies reported here. One notable difference between the findings in the earlier studies of mutational spectra in BD-, BDO-, and BDO_2-exposed preweanling mice and the current inhalation studies in BD- and BDO_2-exposed mice and rats is that the +G frameshift mutation (bp 207–212) that appeared as a putative mutagenic "hotspot" for BD and its epoxy metabolites in preweanling mice was seen only once in BD-exposed mice in the current study. Parallel mutational spectra studies have also been conducted in B6C3F_1 *lacI* transgenic mice exposed to BD (reviewed in Himmelstein et al. 1997; Recio et al. 1997), BDO, and BDO_2. The mice exposed to BDO or BDO_2 for the *lacI* studies were placed in the same exposure chambers as those used by conventional mice in the current *hprt* studies, and it is premature to compare the resulting *lacI* and *hprt* mutational spectra data for these epoxy compounds. The major findings in mice exposed by inhalation to 1,250 ppm BD for 4 weeks relative to findings in air controls were increased frequencies of point mutations at A•T base pairs in bone marrow and spleen and an increased frequency of specific base substitutions at G•C base pairs (that is, G•C→A•T transitions at non-CpG sites and G•C→T•A transversions) in spleen. The predominant mutations in BD-exposed transgenic mice were A•T→T•A transversions. Thus, both the *lacI* and *hprt* mutational spectra data indicate that in vivo exposure to BD induces point mutations at both A•T and G•C base pairs.

A close look at the mutational spectra data obtained by RT-PCR analysis of mutant T cell clones from BD-exposed mice (Table 11) suggests that induced mutations of several classes in *hprt* would have been identified as statistically significant if the number of samples evaluated had been sufficiently large. The average *hprt* MF in exposed mice was 4-fold above background MF, suggesting that 75% of the mutant clones were associated with BD exposure, and that the increased frequency of large deletions alone (19/63) was not sufficient to account for the expected number of induced mutations (47/63). Therefore, more meaningful comparisons could be made to the point mutations identified in *hprt* exon 3 and in the *lacI* gene of BD-exposed mice if the data set for BD-induced mutations over the entire *hprt* gene were expanded in future studies.

The methods recently developed for expanding mutant T cell clones from rodents (Chen et al. 1998; Meng et al. 1998b) and the initial examination of mutations occurring over the *hprt* gene of unexposed and BD-exposed mice lay the groundwork for a more complete analysis of mutations produced by BD and its metabolites in both the mouse and the rat. In general, BD and its epoxy metabolites are relatively weak inducers of gene mutation but are potent inducers of clastogenic effects (reviewed in Himmelstein et al. 1997) and, thus, the carcinogenic properties of BD may be related more to the ability of its metabolites to cause chromosomal deletions and rearrangements than to produce point mutations. Stronger conclusions concerning species similarities and differences in genetic risk may be drawn by the future application of methods allowing the detection and characterization of both large- and small-scale DNA damage occurring over the *hprt* gene of mice, rats, and persons exposed to BD.

IMPLICATION OF FINDINGS

These studies indicate that the novel approach of measuring mutagenic potencies as the change in *hprt* MFs over time in the T cells of exposed animals and controls can be valuable for estimating species differences in mice and rats in mutagenic response to BD. Furthermore, the approach can be useful in predicting the potential role of BDO_2 and other BD-derived metabolites in each species. The resulting *hprt* MF data provide the first evidence that BD is mutagenic in the rat, although the mutagenic response was significantly less than that observed in similarly exposed mice of nearly identical age. Howbeit, the diminished mutagenic response in rats compared with mice is in accordance with the results of rodent cancer bioassays of BD. The magnitude of the species differences in mutagenic potency resembles the species differences in metabolism more closely than the species differences in cancer potency, but the MF data from the two exposure-duration studies in BD-exposed mice and rats suggest that the species differences in mutagenic response would continue to diverge with extended exposures. For example, the species differences in estimated mutagenic potencies in mice compared with potencies in rats increased from 4.4 in animals exposed to 1,250 ppm BD for 2 weeks (Table 1) to 8.5 in animals exposed to 625 ppm BD for 4 weeks (Table 4). Moreover, the estimated mutagenic potencies in splenic T cells for mice exposed to 1,250 ppm BD for 2 weeks (Table 1) were similar to those determined for mice exposed to 625 ppm BD for 4 weeks (Table 4), suggesting that both exposure concentration and exposure duration are important in determining the magnitude of the mutagenic response to BD. Therefore, starting with the quantitative differences in BD metabolism in mice and rats, it is possible that with long-term exposures

the species differences in mutagenic potency become sufficiently large to account for much of the significant species differences observed in cancer potency. Finally, these mutagenic potency studies demonstrate that defining the change in MFs over time, compared with measuring MFs at one point in time, is advantageous because the ratio of the MF measurements in mice compared with rats at a single time point after exposure tends to overestimate or underestimate the species differences in the mutagenic response to BD.

Comparisons of the dose-response and mutagenic-potency data for animals exposed to BD, (±)-BDO, or (±)-BDO_2 at comparable internal doses indicate that BDO_2 is a major contributor to the mutagenicity of the parent compound at lower exposure concentrations of BD (≤ 62.5 ppm) in the mouse, whereas the available metabolism data and *hprt* MF data suggest that other metabolites (the *meso*-BDO_2 and BDO-diol examples discussed below) are responsible both for mutations in BD-exposed rats and for the incremental mutagenic effects at higher concentration exposures in the mouse. The *hprt* exon 3 mutational spectra reported here appear to agree with the extant metabolism and mutagenic potency data as well.

The development of techniques for the large-scale propagation of *hprt* mutant T cell clones from mice and rats provides a means to screen the entire *hprt* gene for large deletions presumably associated mainly with the formation in vivo of (±)-BDO_2, or *meso*-BDO_2, or both. Indeed, the analysis of a substantial set of expanded mutant clones from control and BD-exposed mice, conducted as part of the current work, demonstrated an increased frequency of large deletions following BD exposure. Additional studies to characterize both BD-induced small- and large-scale mutations across the *hprt* gene of the mouse and rat, as is being done for human T cells in a transitional epidemiologic study (Albertini and Šrám 1998), may provide a unique opportunity to compare mutational spectra data for the same target gene in mice, rats, and humans. Comparing lymphocyte *hprt* mutational spectra in BD-exposed humans and rodents may be a good way of evaluating the utility of the rodent models for predicting risk of BD exposure in people. However, comparisons of dose-response relations using *hprt* MFs in T cells are likely to be complicated by species- and age-related differences in thymocyte kinetics (that is, rodents are probably a better quantitative model for children than for human adults because of the shared presence of thymic activity).

Although considerable knowledge has been gained from the various experiments evaluating the mutagenicity of BD and racemic mixtures of BDO and BDO_2, additional work is needed to determine more definitively the relative contribution of these and other BD metabolites (in their various stereochemical configurations) to the DNA alkylation and mutation patterns observed in mice and rats. Accumulated data suggest potential roles for *meso*-BDO_2 and BDO-diol in BD-induced mutagenicity, and priority should be given to these metabolites in future investigations of the effects of BD metabolites in rodent models. Such studies will be important for the delineation of the mutagenic and carcinogenic mechanisms of BD in rodents, for the interpretation of biomarker data obtained from BD workers, and for extrapolation of cancer risk to persons exposed to comparatively low concentrations of BD.

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REFERENCES

Aidoo A, Lyn-Cook LE, Heflich RH, George EO, Casciano DA. 1993. The effect of time after treatment, treatment schedule and animal age on the frequency of 6-thioguanine-resistant T-lymphocytes induced in Fischer 344 rats by *N*-ethyl-*N*-nitrosourea. Mutat Res 298:169–178.

Aidoo A, Lyn-Cook LE, Lensing S, Wamer W. 1994. Ascorbic acid (vitamin C) modulates the mutagenic effects produced by an alkylating agent in vivo. Environ Mol Mutagen 24:220–228.

Aidoo A, Lyn-Cook LE, Mittelstaedt PA, Heflich RH, Casciano DA. 1991. Induction of 6-thioguanine-resistant lymphocytes in Fischer 344 rat following in vivo exposure to *N*-ethyl-*N*-nitrosourea and cyclophosphamide. Environ Mol Mutagen 17:141–151.

Aidoo A, Morris SM, Casciano DA. 1997. Development and utilization of the rat lymphocyte *hprt* mutation assay. Mutat Res 387:69–88.

Albertini RJ, Šrám RJ. 1998. Biomarker responses in butadiene-exposed Czech workers: A transitional epidemiological study (abstract). In: The Fourteenth Health Effects Institute Annual Conference Program and Abstracts, April 5–7, 1998, Boston, MA. Health Effects Institute, Cambridge, MA.

Beland FA. 1995. DNA Adduct Formation and T Lymphocyte Mutation Induction in F344 Rats Implanted with Tumorigenic Doses of 1,6-Dinitropyrene. Research Report Number 72. Health Effects Institute, Cambridge, MA.

Birnbaum LS, Keller D. 1996. Metabolism and pharmacokinetics of 1,3-butadiene. Toxicology 113:14–16.

Blair IA, Kambouris SJ, Oe T, Chaudhary AK, Selzer RR, Elfarra AA, Recio L. 1998. Biomarkers for quantitation of 1,3-butadiene induced DNA damage in B6C3F_1 mice and F344 rats (abstract). In: The Fourteenth Health Effects Institute Annual Conference Program and Abstracts, April 5–7, 1998, Boston, MA. Health Effects Institute, Cambridge, MA.

Bond JA, Recio L, Andjelkovich D. 1995. Epidemiological and mechanistic data suggest that 1,3-butadiene will not be carcinogenic to humans at exposures likely to be encountered in the environment or workplace. Carcinogenesis 16:165–171.

Boogaard PJ, Bond JA. 1996. The role of hydrolysis in the detoxification of 1,2,3,4-diepoxybutane by human, rat, and mouse liver and lung in vitro. Toxicol Appl Pharmacol 141:617–627.

Boogaard PJ, Sumner SCJ, Bond JA. 1996. Glutathione conjugation of 1,2:3,4-diepoxybutane in human liver and mouse and rat liver and lung in vitro. Toxicol Appl Pharmacol 136:307–316.

Carr GJ, Gorelick NJ. 1996. Mutational spectra in transgenic animal research: Data analysis and study design based upon mutant or mutation frequency. Environ Mol Mutagen 28:405–413.

Chen T, Aidoo A, Casciano DA, Heflich RH. 1998. Expansion of rat 6–thioguanine-resistant T-lymphocyte clones by stimulation with ionomycin and a phorbol ester. Environ Mol Mutagen 31:97–102.

Cochrane JE, Skopek TR. 1994a. Mutagenicity of butadiene and its epoxide metabolites: I. Mutagenic potential of 1,2-epoxybutene, 1,2,3,4-diepoxybutane and 3,4-epoxy-1,2-butanediol in cultured human lymphoblasts. Carcinogenesis 15:713–718.

Cochrane JE, Skopek TR. 1994b. Mutagenicity of butadiene and its epoxide metabolites: II. Mutational spectrum of butadiene, 1,2-epoxybutene and diepoxybutane at the *hprt* locus in splenic T cells from exposed B6C3F1 mice. Carcinogenesis 15:719–723.

Cunningham MJ, Choy WN, Arce GT, Rickard LB, Vlachos DA, Kinney LA, Sariff AM. 1986. In vivo sister chromatid exchange and micronucleus induction studies with 1,3-butadiene in B6C3F1 mice and Sprague-Dawley rats. Mutagenesis 1:449–452.

Delzell E, Sathiakumar N, Hovinga M, Macaluso M, Julian J, Larson R, Cole P, Muir DCF. 1996. A follow-up study of synthetic rubber workers. Toxicology 113:182–189.

Divine BJ, Hartman CM. 1996. Mortality update of butadiene production workers. Toxicology 113:169–181.

Divine BJ, Wendt JK, Hartman CM. 1993. Cancer mortality among workers at a butadiene production facility. In: Butadiene and Styrene: Assessment of Health Hazards (Sorsa M, Peltonen K, Vainio H, Hemminki K, eds.). International Agency for Research on Cancer, Lyon, France.

Downs TD, Crane MM, Kim KW. 1987. Mortality among workers at a 1,3-butadiene facility. Am J Ind Med 13:311–329.

Elfarra AA, Krause RJ, Selzer RR. 1996. Biochemistry of 1,3-butadiene metabolism and its relevance to 1,3-butadiene-induced carcinogenicity. Toxicology 113:23–30.

Heddle JA. 1999. Mutant manifestations: The time factor in somatic mutagenesis. Mutagenesis 14:1–3.

Henderson RF, Benson JM, Belinsky SA, Barr EB, Hahn FF. 1998. Carcinogenicity of butadiene diepoxide in B6C3F1 mice and Sprague-Dawley rats (abstract). In: The Fourteenth Health Effects Institute Annual Conference Program and Abstracts, April 5–7, 1998, Boston, MA. Health Effects Institute, Cambridge, MA.

Henderson RF, Benson JM, Hahn FF, Barr EB, Bechtold WE, Burt DG, Dahl AR. 1997. Acute toxicity of butadiene in B6C3F1 mice and Sprague-Dawley rats. Toxicologist 36:177.

Henderson RF, Thornton-Manning JR, Bechtold WE, Dahl AR. 1996. Metabolism of 1,3-butadiene: Species differences. Toxicology 113:17–22.

Himmelstein MW, Acquavella JP, Recio L, Medinsky MA, Bond JA. 1997. Toxicology and epidemiology of 1,3-butadiene. CRC Crit Rev Toxicol 27:1–108.

Himmelstein MW, Asgharian B, Bond JA. 1995. High concentrations of butadiene epoxides in livers and lungs of mice compared to rats exposed to 1,3-butadiene. Toxicol Appl Pharmacol 132:281–288.

Himmelstein MW, Turner MJ, Asgharian B, Bond JA. 1994. Comparison of blood concentrations of 1,3-butadiene and butadiene epoxides in mice and rats exposed to 1,3-butadiene by inhalation. Carcinogenesis 15:1479–1486.

Himmelstein MW, Turner MJ, Asgharian B, Bond JA. 1996. Metabolism of 1,3-butadiene: Inhalation pharmacokinetics and tissue dosimetry of butadiene epoxides in rats and mice. Toxicology 113:306–309.

Hirokawa K, Makinodan T. 1975. Thymic involution: Effect on T cell differentiation. J Immunol 114:1659–1664.

Hollander M, Wolfe DA. 1973. Nonparametric Statistical Methods. Wiley & Sons, New York, NY.

Huff JE, Melnick RL, Solleveld HA, Haseman JK, Powers M, Miller RA. 1985. Multiple organ carcinogenicity of 1,3-butadiene in B6C3F1 mice after 60 weeks of inhalation exposure. Science 227:548–549.

Jacobson-Kram D, Rosenthal SL. 1995. Molecular and genetic toxicology of 1,3-butadiene. Mutat Res 339:121–130.

Jones IM, Burkhart-Schultz K, Strout CL, Carrano AV. 1985a. A method to quantify spontaneous and in vivo induced thioguanine-resistant mouse lymphocytes. Mutat Res 147:97–105.

Jones IM, Burkhart-Schultz K, Strout CL, Carrano AV. 1985b. A study of the frequency of sister-chromatid exchange and of thioguanine-resistant cells in mouse spleen lymphocytes after in vivo exposures to ethylnitrosourea. Mutat Res 143:245–249.

Jones IM, Burkhart-Schultz K, Strout CL, Crippen TL. 1987a. Cloned mouse lymphocytes permit analysis of somatic mutations that occur in vivo. Somat Cell Mol Genet 13:325–333.

Jones IM, Burkhart-Schultz K, Strout CL, Crippen TL. 1987b. Factors that affect the frequency of thioguanine-resistant lymphocytes in mice following exposure to ethylnitrosourea. Environ Mutagen 9:317–329.

Koc H, Tretyakova NY, Walker VE, Swenberg JA. 1999. Molecular dosimetry of N-7-guanine adduct formation in mice and rats exposed to 1,3-butadiene. Chem Res Toxicol 12:566–574.

Krause RJ, Elfarra AA. 1997. Oxidation of butadiene monoepoxide to *meso*- and (±)-diepoxybutane by cDNA-expressed human cytochrome P450s and by mouse, rat, and human liver microsomes: Evidence for preferential hydration of *meso*-diepoxybutane in rat and human liver microsomes. Arch Biochem Biophys 337:176–184.

Landrigan PJ. 1993. Critical assessment of epidemiological studies on the carcinogenicity of 1,3-butadiene and styrene. In: Butadiene and Styrene: Assessment of Health Hazards (Sorsa M, Peltonen K, Vainio H, Hemminki K, eds.). International Agency for Research on Cancer, Lyon, France.

Medinsky MA, Leavens TL, Csanady GA, Gargas ML, Bond JA. 1994. In vivo metabolism of butadiene by mice and rats: A comparison of physiological model predictions and experimental data. Carcinogenesis 15:1329–1340.

Melnick RL, Huff J. 1992. 1,3-Butadiene: Toxicity and carcinogenicity in laboratory animals and in humans. Rev Environ Contam Toxicol 124:111–144.

Melnick RL, Huff J, Chou BJ, Miller R. 1990. Carcinogenicity of 1,3-butadiene in C57BL/6×C3H F1 mice at low exposure concentrations. Cancer Res 50:6592–6599.

Melnick RL, Kohn MC. 1995. Mechanistic data indicate that 1,3-butadiene is a human carcinogen. Carcinogenesis 16:157–163.

Melton DW, Konecki DS, Brennard J, Caskey CT. 1984. Structure, expression, and mutation of the hypoxanthine-guanine phosphoribosyltransferase gene. Proc Natl Acad Sci USA 81:2147–2151.

Meng Q, Henderson RF, Walker VE. 1997. Mutagenicity at *hprt* in splenic T-cells of mice and rats exposed by inhalation to diepoxybutane. Toxicologist 36:308.

Meng Q, Recio L, Reilly AA, Wong B, Bauer M, Walker VE. 1998a. Comparison of the mutagenic potency of 1,3-butadiene at the *hprt* locus of lymphocytes following inhalation exposure of female B6C3F1 mice and F344 rats. Carcinogenesis 19:1019–1027.

Meng Q, Singh N, Heflich RH, Walker VE. 2000. Comparison of the mutations at *Hprt* exon 3 of T-lymphocytes from

B6C3F1 mice and F344 rats exposed by inhalation to 1,3-butadiene or 1,2:3,4-diepoxybutane. Mutat Res 464:(in press).

Meng Q, Skopek TR, Walker DM, Hurley-Leslie S, Chen T, Zimmer DM, Walker VE. 1998b. Culture and propagation of *Hprt* mutant T-lymphocytes isolated from mouse spleen. Environ Mol Mutagen 32:236–243.

Mittelstaedt RA, Smith BA, Heflich RH. 1995. Analysis of in vivo mutation induced by *N*-ethyl-*N*-nitrosourea in the *hprt* gene of rat lymphocytes. Environ Mol Mutagen 26:261–269.

National Toxicology Program. 1984. Toxicology and Carcinogenesis Studies of 1,3-Butadiene in B6C3F1 mice. NTP TR No. 288. National Toxicology Program, U.S. Department of Health and Human Services, Research Triangle Park, NC.

Nieusma JL, Claffey DJ, Maniglier-Poulet C, Imiolczyk T, Ross D, Ruth JA. 1997. Stereochemical aspects of 1,3-butadiene metabolism and toxicity in rat and mouse liver microsomes and freshly isolated rat hepatocytes. Chem Res Toxicol 10:450–456.

Oe T, Kambouris SJ, Walker VE, Meng Q, Recio L, Wherli S, Chaudhary AK, Blair IA. 1999. Persistence of 1,3:3,4-diepoxybutane N-7-guanine adducts in the tissue of mice exposed to 1,3-butadiene compared with rats. Chem Res Toxicol 12:247–257.

Owen PE, Glaiser JR, Gaunt IF, Pullinger DH. 1987. Inhalation toxicity studies with 1,3-butadiene: 3. Two-year toxicity/carcinogenicity studies in rats. Am Ind Hyg Assoc J 48:407–413.

Paulson AS, Delehanty TA. 1982. Sensitivity analysis in experimental design. In: Computer Science and Statistics: Proceedings of the 14th Symposium on the Interface (Heiner KW, Sacher RS, Wilkinson JW, eds.). Springer-Verlag, New York, NY.

Press WH, Flannery BP, Teukolsky SA, Vetterling WT. 1992. The Art of Scientific Computing. Cambridge University Press, New York, NY.

Recio L, Henderson RF, Pluta LJ, Meyer K, Saranko CJ, Steen A-M. 1998. Analysis of mutagenicity and determination of mutational spectrum in rodent and human cells to assess the roles of epoxybutene and diepoxybutane in mediating the in vivo genotoxicity of 1,3-butadiene (abstract). In: The Fourteenth Health Effects Institute Annual Conference Program and Abstracts, April 5–7, 1998, Boston, MA. Health Effects Institute, Cambridge, MA.

Recio L, Meyer KG. 1995. Increased frequency of mutations at A:T base pairs in the bone marrow of B6C3F1 lacI transgenic mice exposed to 1,3-butadiene. Environ Mol Mutagen 26:1–8.

Recio L, Meyer KG, Pluta LJ, Moss OR, Saranko CJ. 1996a. Assessment of 1,3-butadiene mutagenicity in bone marrow of B6C3F1 lacI transgenic mice (Big Blue®): A review of mutational spectrum and lacI mutant frequency after a 5-day 625 ppm 1,3-butadiene exposure. Environ Mol Mutagen 28:424–429.

Recio L, Sisk S, Meyer K, Pluta L, Bond JA. 1996b. Mutagenicity and mutational spectra of 1,3-butadiene in bone marrow of B6C3F1 *lacI* transgenic mice. Toxicology 113:106–111.

Recio L, Steen A-M, Bond JA. 1997. Linking dosimetry of butadiene with mutational spectra to assess the role of epoxybutene and diepoxybutane in the genotoxicity of butadiene. CIIT Activities 17(8):1–9.

Santos-Burgoa C, Matanoski GM, Zeger S, Schwartz L. 1992. Lympho-hematopoietic cancer in styrene-butadiene polymerization workers. Am J Epidemiol 136:843–854.

Saranko CJ, Pluta LJ, Recio L, Henderson RF. 1998. In vivo and in vitro mutagenicity spectrum of the butadiene metabolite 1,2-epoxybutene. Proc Am Assoc Cancer Res 39:330.

Sisk S, Pluta LJ, Bond JA, Recio L. 1994. Molecular analysis of lacI mutants from bone marrow of B6C3F1 transgenic mice following inhalation exposure to 1,3-butadiene. Carcinogenesis 15:471–477.

Skopek TR. 1995. Of mice and mutants: Target size and sensitivity. Mutat Res 331:225–228.

Skopek TR, Kort KL, Marino DR. 1995. Relative sensitivity of the endogenous *hprt* gene and *lacI* transgene in ENU-treated Big Blue™ mice. Environ Mol Mutagen 26:9–15.

Skopek TR, Kort KL, Marino DR, Mittal LV, Umbenhauer DR, Laws GM, Adams SP. 1996. Mutagenic response of the endogenous *hprt* gene and *lacI* transgene in benzo[a]pyrene-treated Big Blue® mice. Environ Mol Mutagen 28:376–384.

Skopek TR, Walker VE, Cochrane JE, Craft TR, Cariello NF. 1992. Mutational spectrum of *N*-ethyl-*N*-nitrosourea at the *hprt* locus in splenic T-cells of exposed B6C3F1 mice. Proc Natl Acad Sci USA 89:7866–7870.

Sollay RG, Butcher EC, Weissman IL. 1980. Thymus cell migration: Quantitative aspects of cellular traffic from the thymus to the periphery in mice. Eur J Immunol 10: 210–218.

Sorsa M, Autio K, Demopoulos NA, Järventaus H, Rössner P, Šrám RJ, Staphanou G, Vlachodimitropoulòs D. 1994.

Human cytogenetic biomonitoring of occupational exposure to 1,3-butadiene. Mutat Res 309:321–326.

Sorsa M, Peltonen K, Anderson D, Demopoulos NA, Neumann H-G, Osterman-Golkar S. 1996. Assessment of environmental and occupational exposures to butadiene as a model for risk estimation of petrochemical emissions. Mutagenesis 11:9–17.

Šrám RJ, Rössner P, Peltonen K, Podrazilová K, Mracková G, Demopoulos NA, Stephanou G, Vladimiropoulos D, van Dam FJ, Tates AD. 1998. Chromosomal aberrations, sister-chromatid exchanges, cells with high frequency of SCE, micronuclei and comet assay parameters in 1,3-butadiene-exposed workers. Mutat Res 419:145–154.

Steen A-M, Meyer KG, Recio L. 1997a. Analysis of *hprt* mutations occurring in human TK6 lymphoblastoid cells following exposure to 1,2,3,4-diepoxybutane. Mutagenesis 12:61–67.

Steen A-M, Meyer KG, Recio L. 1997b. Characterization of *hprt* mutations following 1,2-epoxy-3-butene exposure of human TK6 cells. Mutagenesis 12:359–364.

Swenberg JA, Christova-Gueorguieva NI, Scheller N, Ranasinghe A. 1998. Quantitation of epoxybutene and trihydroxybutane hemoglobin adducts within exposed and control mice, rats and humans (abstract). In: The Fourteenth Health Effects Institute Annual Conference Program and Abstracts, April 5–7, 1998, Boston, MA. Health Effects Institute, Cambridge, MA.

Tan RS, Chu EHY, Sinsheimer JE. 1993. Molecular analysis of hypoxanthine phosphoribosyltransferase mutants induced by glycidyl 1-naphthyl ether in mouse spleen cells in vivo. Environ Mol Mutagen 22:71–77.

Tates AD, van Dam FJ, de Zwart FA, van Teylingen CMM, Natarajan AT. 1994. Development of a cloning assay with high cloning efficiency to detect induction of 6-thioguanine-resistant lymphocytes in spleen of adult mice following in vivo inhalation exposure to 1,3-butadiene. Mutat Res 309:299–306.

Tates AD, van Dam FJ, de Zwart FA, Darroudi F, Natarajan AT, Rossner P, Peterkova K, Peltonen K, Demopoulos NA, Stephanou G, Vlachodimitropoulos D, Šrám RJ. 1996. Biological effect monitoring in industrial workers from the Czech Republic exposed to low levels of butadiene. Toxicology 113:91–99.

Tates AD, van Dam FJ, van Teylingen CMM, de Zwart FA, Zwinderman AH. 1998. Comparison of induction of hprt mutations by 1,3-butadiene and/or its 1,2-epoxybutene and 1,2,3,4-diepoxybutane in lymphocytes from spleen of adult male mice and rats in vivo. Mutat Res 397:21–36.

Thornton-Manning JR, Dahl AR, Allen ML, Bechtold WE, Griffith WC Jr, Henderson RF. 1998. Disposition of butadiene epoxides in Sprague-Dawley rats following exposures to 8,000 ppm 1,3-butadiene: Comparisons with tissue epoxide concentrations following low-level exposures. Toxicol Sci 41:167–173.

Thornton-Manning JR, Dahl AR, Bechtold WE, Griffith WC Jr, Henderson RF. 1995a. Disposition of butadiene monoepoxide and butadiene diepoxide in various tissues of rats and mice following a low-level inhalation exposure to 1,3-butadiene. Carcinogenesis 16:1723–1731.

Thornton-Manning JR, Dahl AR, Bechtold WE, Griffith WC Jr, Pei L, Henderson RF. 1995b. Gender differences in the metabolism of 1,3-butadiene in Sprague-Dawley rats following a low-level inhalation exposure. Carcinogenesis 16:2875–2878.

Thornton-Manning JR, Dahl AR, Bechtold WE, Henderson RF. 1996. Gender and species differences in the metabolism of 1,3-butadiene to butadiene monoepoxide and butadiene diepoxide in rodents following low-level inhalation exposures. Toxicology 113:322–325.

Tretyakova NY, Chiang SU, Walker VE, Swenberg JA. 1998a. Quantitative analysis of 1,3-butadiene-induced DNA adducts in vivo and in vitro using LC-EST+MS/MS. J Mass Spectrom 33:363–376.

Tretyakova NY, Walker VE, Swenberg JA. 1998b. Formation and persistence of DNA adducts in liver of rats and mice exposed to 1,3-butadiene. Toxicologist 42:180.

Walker VE, Andrews JL, Clement NL, Upton PB, deBoer JG, Sussman HE, Gorelick NJ. 1999. Detection of cyclophosphamide-induced mutations at the *hprt* but not the *lacI* locus in splenic lymphocytes of exposed mice. Environ Mol Mutagen 34:167–181.

Walker VE, Gorelick NJ, Andrews JL, Craft TR, deBoer JG, Glickman BW. 1996. Frequency and spectrum of ethylnitrosourea-induced mutation at the *hprt* and *lacI* loci in splenic lymphocytes of exposed *lacI* transgenic mice. Cancer Res 56:4654–4661.

Walker VE, Sisk SC, Upton PB, Wong BA, Recio L. 1997. In vivo mutagenicity of ethylene oxide at the *hprt* locus in T-lymphocytes of B6C3F1 *lacI* transgenic mice following inhalation exposure. Mutat Res 392:211–222.

Walker VE, Skopek TR. 1993a. A mouse model for the study of in vivo mutational spectra: Sequence specificity of ethylene oxide at the *hprt* locus. Mutat Res 288:151–162.

Walker VE, Skopek TR. 1993b. Age-dependent relationships between exposures and phenotypic expression of *hprt* mutations in T-cells from mice treated with *N*-ethyl-*N*-nitrosourea. Environ Mol Mutagen 21(Suppl 22):75.

Walker VE, Walker DM. 1997. Mutagenicity at the *hprt* locus of T-cells following drinking water exposures of F344 rats to acrylonitrile. Toxicologist 36:308.

Ward JB Jr, Ammenheuser MM, Bechtold WE, Whorton EB Jr, Legator MS. 1994. *HPRT* mutant lymphocyte frequencies in workers at a 1,3-butadiene production plant. Environ Health Perspect 102:79–85.

Ward JB Jr, Ammenheuser MM, Bechtold WE, Whorton EB Jr, Legator MS. 1996a. Assessment of butadiene exposure in a rubber plant using a urine metabolite and hprt mutant frequency as biological markers of exposure and effect. Environ Mol Mutagen 27(Suppl 27):73.

Ward JB Jr, Ammenheuser MM, Whorton EB Jr, Bechtold WE, Kelsey KT, Legator MS. 1996b. Biological monitoring for mutagenic effects of occupational exposures to butadiene. Toxicology 113:84–90.

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PUBLICATIONS RESULTING FROM THIS RESEARCH

Meng Q, Recio L, Wong B, Walker VE. 1996. Comparison of the mutagenic potency of butadiene at *hprt* of T-cells following inhalation exposures of B6C3F1 mice and F344 rats (abstract). Environ Mol Mutagen 27(Suppl 27):48.

Meng Q, Henderson RF, Walker VE. 1997. Mutagenicity at *hprt* in splenic T-cells of mice and rats exposed by inhalation to diepoxybutane (abstract). Toxicologist 36(Suppl 1):308.

Meng Q, Walker VE. 1997. Comparison of mutational spectra in *hprt* exon 3 of T-cells from B6C3F1 mice and F344 rats exposed by inhalation to 1,3-butadiene (abstract). Environ Mol Mutagen 29(Suppl 28):36.

Tretyakova NY, Chang S-Y, Walker V, Hass JR, Swenberg JA. 1997. DNA adducts in TK6 cells and tissues of rats and mice exposed to 1,3-butadiene and its metabolites (abstract). Proc Am Assoc Cancer Res 38:333.

Walker VE, Meng Q, Clement NL. 1997. Spectra of mutations in *hprt* exon 3 of T-cells from F344 rats and *lacI* transgenic and nontransgenic B6C3F1 mice exposed by inhalation to ethylene oxide (abstract). Environ Mol Mutagen 29(Suppl 28):54.

Meng Q, Henderson RF, Reilly AA, Bauer M, Walker VE. 1998. Relative contribution of epoxybutene versus diepoxybutane to the mutagenicity of butadiene following inhalation exposures of mice and rats (abstract). Toxicologist 42(Suppl 1-S):182.

Meng Q, Recio L, Reilly AA, Wong BA, Bauer M, Walker VE. 1998. Comparison of the mutagenic potency of 1,3-butadiene at the *hprt* locus of T-lymphocytes following inhalation exposure of female B6C3F1 mice and F344 rats. Carcinogenesis 19:1019–1027.

Meng Q, Skopek TR, Walker DM, Hurley-Leslie S, Chen T, Zimmer DM, Walker VE. 1998. Culture and propagation of *hprt* mutant T-lymphocytes isolated from mouse spleen. Environ Mol Mutagen 32:236–243.

Meng Q, Walker VE. 1998. Characterization of *hprt* mutations in T-lymphocytes from B6C3F1 mice and F344 rats exposed to 1,3-butadiene or diepoxybutane (abstract). Proc Am Assoc Cancer Res 39:184.

Meng Q, Walker VE. 1998. Propagation of *hprt* mutant T-lymphocytes from mice exposed to 1,3-butadiene (abstract). Environ Mol Mutagen 31(Suppl 29):46.

Tretyakova NY, Chiang SU, Walker VE, Swenberg JA. 1998. Quantitative analysis of 1,3-butadiene-induced DNA adducts in vivo and in vitro using LC-EST+MS/MS. J Mass Spectrom 33:363–376.

Tretyakova NY, Walker VE, Swenberg JA. 1998. Formation and persistence of DNA adducts in liver of rats and mice exposed to 1,3-butadiene (abstract). Toxicologist 42(Suppl 1-S):180.

Walker VE, Chang PY, Winegar RA, Walker DM, Reilly AA, Bauer M, Meng Q, Lutze-Mann L. 1998. Mutagenicity of high LET-irradiation at the endogenous *hprt* gene and the lacZ transgene of exposed mice with differing p53 status (abstract). Proc Am Assoc Cancer Res 39:126 (updated).

Koc H, Tretyakova NY, Walker VE, Swenberg JA. 1999. Molecular dosimetry of N-7-guanine adduct formation in mice and rats exposed to 1,3-butadiene. Chem Res Toxicol 12:566–574.

Koivisto P, Walker V, Peltonen K. 1999. Epoxy metabolites of 1,3-butadiene and in vivo DNA alkylation at N7 position of guanine. Proc Am Assoc Cancer Res 40:48.

Meng Q, Chen T, Heflich RH, Walker VE. 1999. Analysis of mutations in *hprt* cDNA of splenic T-lymphocytes from mice exposed by inhalation to 1,3-butadiene. Toxicologist 48(Number 1–5):342.

Meng Q, Henderson RF, Reilly AA, Bauer M, Chen T, Heflich RH, Walker VE. 1999. Mutagenicity of 1,3-butadiene at the *hprt* locus of T-cells following inhalation exposures of female mice and rats. Mutat Res 429:107–125.

Meng Q, Henderson RF, Reilly AA, Bauer M, Walker VE. 1999. Mutagenicity of epoxybutene and diepoxybutane at the *hprt* locus of T-cells following inhalation exposures of mice and rats. Mutat Res 429:127–140.

Oe T, Kambouris SJ, Walker VE, Meng Q, Recio L, Wherli S, Chaudhary AK, Blair IA. 1999. Persistence of 1,3:3,4-diepoxybutane N-7-guanine adducts in the tissue of mice exposed to 1,3-butadiene compared with rats. Chem Res Toxicol 12:247–257.

Meng Q, Singh N, Heflich RH, Walker VE. 2000. Comparison of the mutations at *hprt* exon 3 of T-lymphocytes from B6C3F1 mice and F344 rats exposed by inhalation to 1,3-butadiene or 1,2:3,4-diepoxybutane. Mutat Res 464:(in press).

ABBREVIATIONS AND OTHER TERMS

BD	1,3-butadiene
BDO	1,2-epoxy-3-butene
BDO_2	1,2,3,4-diepoxybutane
BDO-diol	1,2-dihydroxy-3,4-epoxybutane
bp	base pair or pairs
CI	confidence interval
CV	coefficient of variation
DGGE	denaturing gradient gel electrophoresis
hprt	hypoxanthine-guanine phosphoribosyltransferase gene
IL-2	interleukin-2
MF	mutant frequency
NMR	nuclear magnetic resonance (imaging)
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase–polymerase chain reaction

COMMENTARY ON PARTS II AND III
Health Review Committee

HEI

INTRODUCTION

In response to RFA-93-1, *Novel Approaches to Extrapolation of Health Effects for Mobile Source Toxic Air Pollutants*, Dr. Leslie Recio of the Chemical Industry Institute of Toxicology submitted an application entitled "Determination of Mutagenicity and Mutational Spectrum in Human and Rodent Cells to Assess the Role(s) of Butadiene Monoepoxide and Butadiene Diepoxide in Mediating the In Vivo Genotoxicity of 1,3-Butadiene." Recio planned to extend his work on mutagenicity induced in mice by 1,3-butadiene (BD)* (described in the Scientific Background section of this Commentary) by comparing the mutagenicity of two epoxide metabolites of BD in vitro and in vivo. External reviewers and the HEI Health Research Committee considered the strength of Recio's study to be its experimental design, which sought to complete a matrix of comparisons across species and between in vitro and in vivo effects.

Dr. Vernon Walker of the State University of New York in Albany also submitted an application, entitled "1,3-Butadiene Mutagenicity and Tumorigenicity in T Lymphocytes of Exposed Rodents." Walker planned to compare both the degree of BD-induced mutagenicity in rats and mice and the mutant frequency induced by the same epoxide metabolites studied by Recio. Reviewers thought that Walker's proposal to study mutations in the same gene in rats and mice would aid extrapolation between species and was an important step before attempting to extrapolate from rodents to humans.

After considering the comments of external reviewers and the Research Committee, the investigators submitted revised applications. The Committee viewed each favorably because, in addition to their scientific merit, the information on mutagenicity provided by each study would complement that of the other.†

During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Reports and in the Health Review Committee's Commentary. The following Commentary is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the studies, by pointing out alternative explanations for data, and by placing the Investigators' Reports into scientific and regulatory perspective.

The Glossary in the accompanying sidebar defines terms used in this Commentary.

SCIENTIFIC BACKGROUND

MUTAGENICITY OF BD AND ITS EPOXIDE METABOLITES

Multiple mutations are found in cancer cells (Hussain and Harris 1998); such genetic mutations, either spontaneous (background) or induced by chemical exposure, may be important in cancer development. Some chemicals bind to DNA to form DNA adducts, which can cause point mutations through interference in normal DNA replication or repair. The pattern of genetic changes is often specific for a given chemical; the mutational spectrum produced can be considered a fingerprint of exposure to that chemical agent and may thus serve as a biomarker of exposure. For example, BD inhalation causes an increase in point mutations at A•T base pairs in mice (Sisk et al. 1994; Cochrane and Skopek 1994b; Recio and Meyer 1995; Recio et al. 1996).

Most studies of BD-induced mutagenicity have been carried out in mice (reviewed by Himmelstein et al. 1997) because they are more sensitive to tumor induction by BD than are rats. Researchers have focused on the marker genes such as the endogenous hypoxanthine phosphoribosyltransferase (*hprt*) gene or the *lacI* transgene (of bacterial origin) in transgenic mice and rats; they reported an increased mutant frequency in both transgenes and endogenous genes of mice exposed to BD by inhalation (Recio et al. 1992, 1996; Cochrane and Skopek 1994b; Sisk et al. 1994; Tates et al. 1994).

Butadiene is not a direct-acting mutagen. When BD is taken up in the body, many tissues convert it enzymatically to reactive epoxide metabolites, which may react with cellular molecules. Researchers are not certain, however, which of the epoxide metabolites play major roles in BD-induced mutagenicity (see Figure 1 in the Preface to

* A list of abbreviations appears at the end of each Investigators' Report.

† Dr. Leslie Recio's study began in December 1994 and had total expenditures of $474,054. The Investigators' Report from Recio and colleagues was received for review in December 1997. A revised report, received in June 1998, was accepted for publication in August 1998. Dr. Vernon Walker's study began in January 1995 and had total expenditures of $224,205. The Investigators' Report from Walker and Meng was received for review in June 1998. A revised report, received in November 1998, was accepted for publication in December 1998.

GLOSSARY

Epoxide: A reactive molecule containing an oxygen atom joined to each of two carbon atoms bonded to each other

Diepoxide: A reactive molecule containing two epoxides

Racemic Mixture: A compound made up of equal isomers with opposite configurations at one or more asymmetric centers

Transgene: The result of experimentally slicing a segment of DNA from one genome onto DNA of a different genome

Exon: A region of a gene that codes for the structure of a protein; genes contain multiple exons that differ in length

Mutation: A change in the normal sequence of DNA in a gene

Point Mutation: Alteration at a specific base pair within a gene; genes may undergo a wide range of point mutations, including transitions and transversions

Transition Mutation: A mutation caused by a purine base being replaced by a different purine or a pyrimidine base being replaced by a different pyrimidine

Transversion Mutation: A mutation caused by a purine base being replaced by a pyrimidine or a pyrimidine base being replaced by a purine

Deletion Mutation: A mutation caused by loss of one or more DNA base pairs

Insertion Mutation: A mutation caused by addition of one or more DNA base pairs

Mutational Spectrum: Among a set of mutant genes, the pattern of changes observed in the normal sequence of purine or pyrimidine bases that is caused by a mutagen; changes in DNA (mutations) can include type (e.g., point mutations, deletions), location of the mutation in the sequence of bases, and frequency of the specific mutational change

Clastogen: An agent (for example, chemical, x-rays, or ultraviolet light) capable of breaking chromosomal strands

Plasmid: A small, circular DNA molecule that can be incorporated into cells; it can exist outside chromosomes or become integrated into the host DNA

this Research Report for a summary of BD metabolism). Studies by several researchers (Reardon et al. 1987; Gay and Contamine 1993; Cochrane and Skopek 1994a) suggest that the predominant genetic changes produced by exposure to 1,2-epoxy-3-butene (BDO) are point mutations, whereas those produced by exposure to its oxidation product, 1,2,3,4-diepoxybutane (BDO_2), are small and large deletions of DNA base pair segments. Both BDO and BDO_2 increased the *hprt* mutant frequency in splenic T lymphocytes taken from mice exposed to the metabolites by intraperitoneal injection (Cochrane and Skopek 1994b). In vitro studies have shown that BDO_2 is more potent than BDO as an inducer of chromosomal aberrations in animal and human cells and of mutations within the *hprt* gene of human cells (Sasiadek et al. 1991a,b; Cochrane and Skopek 1994a). An important observation was that the concentrations of BDO_2 that were genotoxic in vitro (Cochrane and Skopek 1994a) were similar to the levels of BDO_2 measured in the blood and tissues of mice exposed to BD by inhalation (Himmelstein et al. 1994, 1995; Thornton-Manning et al. 1995). Tates and colleagues (1998) pointed out that differences in experimental design may affect the results of studies with BD metabolites. For example, their finding that BDO_2 did not increase *hprt* mutations in splenic lymphocytes from adult mice contrasts with the positive results of Cochrane and Skopek (1994b), who used 12-day-old mice. Thus, Tates and colleagues (1998) suggested that the response to BDO_2 may vary with age. In addition, the relative mutagenicity of the different stereochemical forms of the BD epoxides (see Preface) must be taken into consideration. Such differences may shed light on the mechanisms of BD-induced carcinogenicity and ultimately lead to improved human risk assessments.

hprt AND *lacI* AS MUTATIONAL TARGET GENES

The *hprt* and *lacI* genes were the focus of the mutational assays. Mutational assays using the *hprt* gene allow researchers to quantify and characterize mutations in the same gene in laboratory animals and in humans. The product of the *hprt* gene is the enzyme hypoxanthine (guanine) phosphoribosyltransferase, which metabolizes the purine bases hypoxanthine and guanine. This enzyme also metabolizes a purine base analog, 6-thioguanine (6-TG) into a toxic intermediate. Normal cells, which produce the enzyme, are killed when 6-TG is added to cell culture medium. In contrast, cells in which the *hprt* gene has mutated to become nonfunctional do grow in the presence of 6-TG because their ability to produce the enzyme has been lost and 6-TG is therefore nontoxic. These cells with mutated *hprt* genes are ideal for identifying the specific

changes in the sequence of bases in the DNA strand for mutational spectrum analysis. Because only a small number of cells containing the mutated gene can be recovered from animals and humans, the number of cells must be expanded by growth in culture. This limits experimental analyses to cells that grow well in vitro (Walker et al. 1996).

Transgenic rodents are used increasingly in genetic toxicology because transgenes are found in all cells; therefore, any tissue can be assayed for mutations. The *lacI* gene is naturally found in certain bacteria, including *Escherichia coli*, and controls the production of the enzyme β-galactosidase, which cleaves the sugar lactose. The *lacI* gene has been used extensively as a target for the identification of mutations, in part due to the ease of using a colorimetric assay to screen rapidly for mutant cells (Kohler et al. 1991). Transgenic rats and mice are produced by injecting embryos with a plasmid containing the bacterial *lacI* gene and additional components that allow the plasmid to reproduce. Transgenic cells are obtained by incorporating the plasmid into cells cultured in vitro. Cell growth is not a limitation because the transgene is recovered from tissue (or from mammalian cells in culture) and transferred to bacterial cells, which can be easily expanded in vitro (Short et al. 1992; Walker et al. 1996).

GOALS OF THE RECIO AND WALKER STUDIES

Goals of the Recio study were to determine the mutagenicity of BDO and BDO_2 in human and rat cells exposed in vitro, as well as in mice and rats exposed in vivo, and to identify the mutational spectrum produced by each metabolite during the in vitro exposures.

The major goal of the Walker study was to test the hypothesis that BD is mutagenic in the rat as well as in the mouse and that differences in the mutagenic potency of BD at the *hprt* locus of rat and mouse T lymphocytes (T cells) can be correlated quantitatively with species differences in BD-induced carcinogenicity. In addition, Walker proposed that comparing the mutagenic potency of BDO and BDO_2 in the *hprt* gene could help to define the relative role of each metabolite in BD-induced mutagenesis. Walker also intended to determine the mutational spectrum at one small part of the *hprt* gene (exon 3) in T cells from rats exposed to BD, BDO, or BDO_2. Because of Walker's methodologic advances in optimizing cell culture techniques, this third goal was later expanded to determine the mutational spectrum across the entire *hprt* gene.

Both studies were integral components of HEI's research program on BD because they sought to characterize the "fingerprint" (mutational spectrum) produced by exposure to epoxide metabolites of BD and to evaluate the contribution of each metabolite to the mutagenic effects of the parent compound.

Table 1 illustrates the endpoints for both studies.

TECHNICAL EVALUATION OF RECIO'S REPORT

Recio's study had three specific aims:

1. determine the mutagenicity and mutational spectra of BDO and BDO_2 in vitro in the *hprt* gene in a human cell line;
2. determine the mutagenicity and mutational spectra of BDO and BDO_2 in vitro in the *lacI* transgene in rat fibroblasts; and
3. determine the mutagenicity of BDO and BDO_2 in tissues from *lacI* transgenic mice and rats exposed by inhalation to BDO or BDO_2.

STUDY DESIGN, METHODS, AND RESULTS

All exposures in the Recio study employed commercially available racemic mixtures of BDO and BDO_2. Recio used state-of-the-art methods in his in vitro and in vivo mutagenicity analyses and in his in vitro mutational spectrum analyses, and he applied appropriate statistical analyses to the data collected.

In Vitro Mutagenicity and Mutational Spectra of BDO and BDO_2 at the *hprt* Gene in a Human Cell Line

Recio determined the mutant frequency in the *hprt* gene of human TK6 lymphoblastoid cells exposed to either BDO, BDO_2, or no additions (controls) and cultured in medium containing 6-TG. Because BDO_2 is significantly mutagenic only at highly cytotoxic concentrations (approximately 10% cell survival [Cochrane and Skopek 1994a]), the investigators selected a combination of concentration and exposure time to achieve this level of toxicity. The experimental conditions for BDO exposures were chosen to ensure that cells were exposed to each of the two metabolites under essentially similar conditions of cytotoxicity. Using similar conditions was critical because mutational spectra may vary with exposure conditions (Chen and Thilly 1996). Comparing the number of growing cell colonies in control cultures and in chemically exposed cultures provided a measure of mutant frequency. The investigators used molecular biology techniques to compare the previously determined sequence of bases in the *hprt* DNA from background mutants in control cultures and from mutant cells induced by exposure to BDO or

Table 1. Endpoints for the Recio and Walker Studies

Exposure Compound	Recio	Walker
In Vivo Exposures[a]		
BD		• *hprt* mutations in T cells from thymus and spleen of rats and mice • Mutational spectra in *hprt* exon 3 from rat and mouse spleen T cell clones • Mutational spectrum across entire *hprt* gene
BDO	• *lacI* mutations in bone marrow and spleen of transgenic rats and mice and in lungs of mice	• *hprt* mutations in rat and mouse spleen T cells
BDO_2	• *lacI* mutations in bone marrow and spleen of transgenic rats and mice	• *hprt* mutations in rat and mouse spleen T cells • Mutational spectra in *hprt* exon 3 from rat and mouse spleen T cell clones
In Vitro Exposures		
BDO	• *hprt* mutations and mutational spectrum in human TK6 cells • *lacI* mutations and mutational spectrum in rat transgenic fibroblasts	
BDO_2	• Same endpoints as with BDO	

[a] In vivo exposures to BD for the Walker study were performed by Drs. Leslie Recio and Rogene Henderson. In vivo exposures to BDO and BDO_2 for both studies were performed by Dr. Henderson.

BDO_2. By this means, they determined the mutational spectra produced by the two BD metabolites.

Both BDO and BDO_2 were mutagenic to human TK6 cells. After exposure to 400 µM BDO, the mutant frequency at the *hprt* gene increased ninefold compared with the frequency following control cell exposure. Sequence analyses indicated that point mutations accounted for 78% of the mutations in the BDO-exposed cells; the remaining DNA lesions were small and large deletions. The BDO-induced mutational spectrum showed significant increases in A•T→G•C transitions, A•T→T•A transversions, and G•C→A•T transitions compared with mutations in control cells. The latter two types of mutations were also produced in vivo by the parent compound, BD, at the *lacI* transgene in mice (Recio and Meyer 1995). Exposure to 4 µM BDO_2 increased mutant frequency at the *hprt* gene in human TK6 cells fivefold over those of the control cells. Most BDO_2-induced mutant cells contained large deletions; only 19% of the mutants from BDO_2-exposed cells had point mutations. The most frequent point mutation was an A•T→T•A transversion.

In Vitro Mutagenicity and Mutational Spectra of BDO and BDO_2 at the *lacI* Transgene in Rat Fibroblasts

Recio compared the mutant frequency at the *lacI* transgene from control rat transgenic fibroblasts and from transgenic fibroblasts exposed to several concentrations of BDO or BDO_2 in culture. He used sequence analysis to compare the *lacI* mutational spectra of mutants from control and BDO-exposed cells.

Treatment of *lacI* transgenic rat fibroblasts with 1,000 µM BDO produced a threefold increase in mutant frequency compared with that of control cells. Sequence analyses showed significant increases in A•T→C•G transversions, A•T→T•A transversions, G•C→T•A transversions, and G•C→A•T transitions. The latter three mutations had also been identified in the *lacI* gene of BD-exposed mice (Recio and Meyer 1995); finding the same pattern of mutations in rats again points to the possible role of the mutational spectrum as a biomarker for BD exposure.

Recio and coworkers did not observe any BDO_2-induced *lacI* mutant cells, possibly due to two factors. First, the elevated level of background mutations in this gene (Skopek 1995) would be expected to yield smaller differences

between control and BDO_2-exposed cells. Second, BDO_2 is a potent clastogen and causes a high prevalence of large deletion mutations, a type of mutation that is not detected by the *lacI* assay used in this study.

In Vivo Mutagenicity of BDO and BDO_2 in Tissues from *lacI* Transgenic Mice and Rats

Dr. Rogene F. Henderson of the Lovelace Respiratory Research Institute exposed transgenic mice and rats (five to seven weeks of age) to 30 ppm BDO or 4 ppm BDO_2 for 6 hours/day, 5 days/week, for 2 weeks. She chose a concentration of BDO that would achieve a blood level equivalent to that observed after exposing mice (the more sensitive rodent species) to 625 ppm BD; the exposure concentration for BDO_2 was chosen to achieve a blood level equivalent to exposing mice to 62.5 ppm BD. Recio and coworkers performed the mutational assays at their laboratory at the Chemical Industry Institute of Toxicology. They determined the mutagenicity of BDO and BDO_2 in the *lacI* transgene isolated from spleen, lung (for BDO only), and bone marrow cells by the method used with *lacI* rat fibroblasts in vitro.

BDO was not mutagenic at the *lacI* transgene in bone marrow or spleen cells isolated from mice exposed by inhalation. However, inhalation of BDO was mutagenic (with a 2.8-fold increase compared with the control level) in cells from mouse lung, a major target organ for BD-induced carcinogenesis in this species. These results may reflect biological differences in tissue sensitivity caused by altered metabolism of BD to BDO or by subsequent BDO disposition. The investigators also reported a significant 1.5-fold increase in *lacI* mutations in bone marrow cells from BDO-exposed rats compared with the mutant frequency in control rats. However, BDO did not elevate *lacI* mutations in rat spleen cells, and effects on rat lung cells were not determined.

BDO_2 was not mutagenic in mouse bone marrow and spleen cells. Exposure of rats to BDO_2 induced a small but significant elevation in *lacI* mutant frequency in bone marrow cells, but not in spleen cells.

DISCUSSION

Recio and colleagues established that BDO and BDO_2 are mutagenic in vitro and in vivo. These results agree with those of other investigators, whose findings have also suggested that BDO and BDO_2 induce genetic damage in mammalian cells through different mechanisms (Cochrane and Skopek 1994a; Walker and Meng, this Research Report). In the current study, exposure to BDO caused point mutations at adenine and guanine sites in the *hprt* and *lacI* genes. Three of five types of mutations involved guanine sites, which indicates that modification of purines, particularly guanine, may be an important mechanism in the mutagenicity of BDO. In contrast, in the case of BDO_2, results at both *hprt* and *lacI* genes indicate that BDO_2 induces primary deletion mutations.

The mutational spectrum induced by BDO in vitro strikingly resembled that found after exposure to its parent compound in vivo. The three types of mutations at the *lacI* transgene—G•C→A•T transitions, G•C→T•A transversions, and A•T→T•A transversions—were induced by in vitro exposure to BDO in this study as well as by in vivo inhalation exposure of mice to BD (Recio and Meyer 1995). In addition, the current study showed that two of these mutations (G•C→A•T transitions and A•T→T•A transversions) were also induced in the *hprt* gene after in vitro exposure to BDO. Thus, the similar mutational spectra produced in vivo by exposure to the parent compound, BD, and in vitro by the metabolite BDO suggest that these changes may prove to be a relevant biomarker of exposure to BD.

Inhaled BDO caused an increase in mutant frequency in mouse lung, a major target organ for BD carcinogenicity. This result also provides evidence that BDO may be an important contributor to the mutagenic action of BD exposure in vivo, although the role of BDO_2 in mutations in this target organ was not evaluated.

Although Recio found a strong consonance in the mutational spectra induced by BD in vivo and its BDO metabolite in vitro, the possible contribution of BDO_2 to BD's observed carcinogenicity should not be ruled out. Both Walker's results (discussed below) and Henderson's positive findings on the carcinogenicity of BDO_2 (also presented in this Research Report) suggest that this metabolite also plays a role in BD-induced carcinogenicity. Although Recio's findings shed light on the contribution of BDO and BDO_2 to the genotoxicity and carcinogenicity of BD exposure, the use of racemic mixtures of the epoxides rather than specific stereoisomers limits interpretation of the study's results.

TECHNICAL EVALUATION OF WALKER'S REPORT

At its origin, Walker's study had four specific aims:

1. determine species differences in the mutagenic potency of BD in the *hprt* gene from thymus and spleen T cells from mice and rats exposed to BD by inhalation;
2. determine the relative contribution of BDO and BDO_2 to BD-induced mutagenesis in the *hprt* gene in T cells

from mice and rats exposed by inhalation to BD or one of the two epoxides;

3. characterize and compare the nature of mutations occurring in exon 3 of the *hprt* gene of T cells from mice and rats exposed to BD, BDO, or BDO_2 by inhalation; and
4. improve cell culture methods for enhanced growth in vitro and for large-scale propagation of *hprt* mutant T cell clones from mice. The success of this aspect of the study led Walker to use these methods in a new aim: to define and compare mutations occurring over the entire *hprt* gene in T cells in control and BD-exposed mice.

STUDY DESIGN, METHODS, AND RESULTS

In general, this was a well-designed study. Much effort was directed at determining the relative potencies of the parent compound, BD, and racemic mixtures of its epoxide metabolites BDO and BDO_2 in 4-week-old rats and mice. Walker and Meng used kinetic modeling and data for BD and metabolite levels in blood and tissues of rats and mice (summarized by Himmelstein et al. 1997) to select exposure doses of the test compounds. However, it is uncertain whether the selected regimens delivered the internal doses that were predicted. Because Walker did not report blood levels of BDO or BDO_2 during or after inhalation exposures, analyses of the observed differences in the mutant frequency of *hprt* in rats and mice are limited by the lack of measured data on internal dose.

The investigators developed an innovative technique for comparing mutagenic potencies (described below), and their improved culture methods enabled them to make significant advances in determining the spectrum of BD-induced *hprt* mutations. They determined *hprt* mutant frequencies in T cells from thymus and spleen as described in the first specific aim of Recio's study.

Species Differences in the Mutagenic Potency of BD

The investigators exposed mice and rats to 0 (controls) or 1,250 ppm BD for 6 hours/day, 5 days/week, for either 1 or 2 weeks. They measured the mutant frequency of *hprt* 2 weeks after each exposure ceased. Furthermore, after one 2-week exposure, they measured mutant frequency immediately after exposure ceased and at additional time points up to 10 weeks after exposure. This method for measuring the mutagenic potencies (that is, as the change in mutant frequency over time) of BD, BDO, and BDO_2 in causing *hprt* mutations in T cells was particularly noteworthy. Measuring mutant frequencies at multiple time points after cessation of exposure and integrating the number of mutants as a function of time is superior to the standard cross-sectional, single-time measurements. Walker provides an excellent discussion of this approach including its strengths, limitations, and some of the underlying assumptions.

The investigators found that both exposure duration and time after cessation of exposure affected the mutant frequency in the two species. First, the mutant frequency was greater in mouse and rat thymic T cells after 2 weeks of BD exposure compared with the frequency after a 1-week exposure. Second, mutant frequency in mouse spleen cells peaked 5 weeks after exposure ceased, and it peaked in rat spleen cells 4 weeks after exposure ceased. In each case, mutant frequencies were greater in mice compared with frequencies in rats. The mutagenic potency of BD was 4.4-fold greater in splenic T cells from mice than from rats after a 2-week exposure to 1,250 ppm BD; it was 8.5-fold greater after exposure of both species to a lower concentration of BD (625 ppm) for 4 weeks.

Relative Contribution of BDO and BDO_2 to BD-Induced Mutagenesis

First, the investigators studied the mutant frequencies induced by BD, BDO, and BDO_2 under several experimental conditions. In an effort to increase the number of *hprt* mutations, Walker and Meng extended their exposure of rats and mice to 625 ppm BD to either 2 or 4 weeks of duration. They also determined the mutant frequency in splenic T cells compared with the frequency in controls 4 weeks after each exposure ceased. In addition, they studied the effects on *hprt* mutant frequency of (1) elapsed time after exposure ceased and (2) exposure concentration of inhaled BD, BDO, or BDO_2. They used their data on the changes in mutant frequency over time to compare the mutagenic potencies of BD, BDO, and BDO_2.

Mutant frequencies in mice exposed to BD were greater in splenic T cells after a 4-week exposure than after a 2-week exposure. In rats, a significant increase was measured only after the 4-week exposure. Mutant frequencies in mice peaked 5 weeks after BD exposure ceased. Mice showed significant dose-dependent increases in mutant frequency after exposures to 20 to 1,250 ppm BD for 2 weeks and to 20 to 625 ppm BD for 4 weeks. In contrast, mutant frequencies increased only in rats exposed to 625 or 1,250 ppm BD (for 2 weeks) and to 625 ppm BD (for 4 weeks). In all cases, mutant frequencies were greater in mice than in rats.

Mice exposed to BDO showed increased mutant frequency and a dose-response effect only at 7 weeks after exposure. BDO exposure had no effect on rats. Both mice and rats exposed to BDO_2 showed a peak mutant frequency 7 weeks after exposure, and in contrast to the data

from BD exposures, BDO_2-induced mutagenicity was greater in rats than in mice. Both species showed a significant dose-response effect from 1 to 7 weeks after exposure, with a slightly greater effect observed in rats.

A key finding in this study was that comparing the mutagenic potency of BD, BDO, and BDO_2 in mice (Table 4 in the Walker Investigators' Report) suggests that BDO_2, rather than BDO, is a major contributor to BD-induced mutagenicity when mice are exposed to low (62.5 ppm) levels of BD (see Walker's Discussion and Conclusions section for a detailed analysis of these comparisons). Similar comparisons could not be made in rats because mutant frequencies were too low (and differences were not statistically significant) to allow calculation of mutagenic potencies. Possibly the internal dose of BDO_2 in this species is too low to have a major mutagenic effect (discussed by Himmelstein et al. 1997).

Mutations in Exon 3 of the *hprt* Gene of T Cells from Mice and Rats Exposed to BD, BDO, or BDO_2

Walker and Meng characterized the mutational spectra in *hprt* exon 3 from control mice and from BD- and BDO_2-exposed mice and rats by sequence analysis. The mutational spectra at exon 3 from BDO-exposed animals could not be studied because there were too few mutants in mice and none in rats.

Exon 3 represents only a small portion of the *hprt* gene, and only a minor percentage of the total gene mutations identified were in this region; nonetheless, the investigators identified spectral differences between mutations in exon 3 from control animals and from BD- or BDO_2-exposed animals. The majority of the BD- and BDO_2-induced exon 3 mutations in mice and rats were point mutations consisting of transitions and transversions at A•T and G•C base pairs. A large overlap was noted in the BD-induced mutational spectra: between species, between BD- and BDO_2-exposed mice, and between BD- and BDO_2-exposed rats with respect to sites with base substitutions, the specific mutations observed at these sites, and the relative occurrence of the most frequently observed base substitutions.

Mutations in the Entire *hprt* Gene from Control and BD-Exposed Mice

When the study began, limited growth of T cells in culture forced the investigators to confine their mutational spectra studies to the small exon 3 region of the *hprt* gene. As the study progressed, however, the investigators developed methods for large-scale propagation of *hprt* mutant T cell clones from mice. Their improved cell culture techniques then allowed them to determine the mutational spectrum over the entire gene in BD-exposed mice.

The pattern of BD-induced mutations across the entire gene differed from that seen at exon 3. Point mutations accounted for only a small percentage of the total BD-induced mutations seen in the entire gene. Instead, the majority of mutations consisted of deletions.

DISCUSSION

Walker and Meng demonstrated the feasibility of examining BD-induced mutations in vivo at the *hprt* gene. Inhalation exposure of rats to BD led to increased mutant frequency in the *hprt* of T cells, albeit with significantly lower efficacy than occurred in mice. These results are consonant with animal bioassay studies that have demonstrated the enhanced sensitivity of mice to BD-related tumorigenesis compared with rats (see the Preface to this Research Report). Comparing the effects of BD, BDO, and BDO_2 exposures on mutant frequency at levels designed to give equivalent internal doses suggests that both metabolites may contribute to the mutagenic activity from exposure to the parent molecule. At low exposure levels of BD, however, where rates of formation of BDO_2 are not saturated, this metabolite may be a particularly important contributor to mutagenesis in the mouse.

Analyses of the mutational spectra of BD and BDO_2 at exon 3 of the *hprt* gene suggested that modifications of purines could be important mutagenic lesions induced by these compounds. However, examining BD-induced mutations over the entire gene indicated that deletion mutations, rather than purine modifications, predominated in the mouse. Deletion mutations point to the conversion of BD to BDO_2, which is capable of causing such deletions by its clastogenic activity.

Although Walker's findings shed light on the contribution of BDO and BDO_2 to the genotoxicity and carcinogenicity of BD, the use of racemic mixtures of the epoxides rather than specific stereoisomers limits interpretation of the study's results.

CONCLUSIONS

Recio and Walker produced solid studies that provide information on the mutagenicity of racemic mixtures of BDO and BDO_2 in cells from rats, mice, and humans. Recio concluded that BDO is more mutagenic than BDO_2 at the *lacI* gene in both rats and mice; however, his assay precluded identifying deletion mutations that might have been induced by BDO_2. Walker concluded that BDO_2 was more mutagenic than BDO in the mouse *hprt* gene. The relative mutagenicity of these compounds at the rat *hprt* gene remains unclear.

Both investigators acknowledge that using racemic mixtures of the epoxides did not replicate the pattern of the different stereoisomers produced by metabolism in vivo. The stereospecific transformation of BD metabolites limits interpretation of the results of exposure to commercially available racemic mixtures of BDO and BDO_2. The apparently lower tumorigenicity of BD in rats compared with that in mice may be related to the specific stereoisomeric forms of the epoxide metabolites produced by metabolism of BD in the two species. The results of these studies provide a starting point for future research; however, they cannot be considered as definitive. The stereoisomeric form or forms that predominate in tissues of BD-exposed laboratory animals must be determined and evaluated for their individual mutagenicities and mutational spectra. Then, comparing the mutational spectra produced by BD exposure in the *hprt* gene in humans exposed either in the workplace or in ambient outdoor air with the spectra produced by BDO or BDO_2 in rodents should increase our understanding of the carcinogenic risk of human exposure to BD.

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REFERENCES

Chen J, Thilly WG. 1996. Mutational spectra vary with exposure conditions: Benzo[*a*]pyrene in human cells. Mutat Res 357:209–217.

Cochrane JE, Skopek TR. 1994a. Mutagenicity of butadiene and its epoxide metabolites: I. Mutagenic potential of 1,2-epoxybutene, 1,2,3,4-diepoxybutane and 3,4-epoxy-1,2-butanediol in cultured human lymphoblasts. Carcinogenesis 15:713–717.

Cochrane JE, Skopek TR. 1994b. Mutagenicity of butadiene and its epoxide metabolites: II. Mutational spectra of butadiene, 1,2-epoxybutene and diepoxybutane at the *hprt* locus in splenic T cells from exposed $B6C3F_1$ mice. Carcinogenesis 15:719–723.

Gay P, Contamine D. 1993. Study of the ref(2)P locus of *Drosophila melanogaster:* II. Genetic studies of the 37DF region. Mol Gen Genet 239:361–370.

Himmelstein MW, Acquavella JF, Recio L, Medinsky MA, Bond JA. 1997. Toxicology of 1,3-butadiene. Crit Rev Toxicol 27:1–108.

Himmelstein MW, Asgharian B, Bond JA. 1995. High concentrations of butadiene epoxides in livers and lungs of mice compared to rats exposed to 1,3-butadiene. Toxicol Appl Pharmacol 132(2):281–288.

Himmelstein MW, Turner MJ, Asgharian B, Bond JA. 1994. Comparison of blood concentrations of 1,3-butadiene and butadiene epoxides in mice and rats exposed to 1,3-butadiene by inhalation. Carcinogenesis 15(8):1479–1486.

Hussain PS, Harris CC. 1998. Molecular epidemiology of human cancer: Contribution of mutation spectra studies of tumor suppressor genes. Cancer Res 58:4023–4037.

Kohler SW, Provost GC, Fieck A, Kretz PL, Bullock WO, Sorge JA, Putman DL, Short JM. 1991. Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice. Proc Natl Acad Sci USA 88:7958–7962.

Reardon JT, Liljestrand-Golden CA, Dusenberry RL, Smith PD. 1987. Molecular analysis of 1,2,3,4-diepoxybutane-induced deletions at the rosy locus of *Drosophila melanogaster*. Genetics 115:323–331.

Recio L, Meyer K. 1995. Increased frequency of mutations at A•T base pairs in the bone marrow of $B6C3F_1$ *lacI* transgenic mice exposed to 1,3-butadiene. Environ Mol Mutagen 26:1–8.

Recio L, Osterman-Golkar S, Csanády GA, Turner MJ, Mhyr B, Moss O, Bond JA. 1992. Determination of mutagenicity in tissues of transgenic mice following exposure to 1,3-butadiene and *N*-ethyl-*N*-nitrosourea. Toxicol Appl Pharmacol 117:58–64.

Recio L, Sisk S, Meyer K, Pluta L, Bond JA. 1996. Mutagenicity and mutational spectra of 1,3-butadiene in the bone marrow of $B6C3F_1$ *lacI* transgenic mice. Toxicology 113:106–111.

Short JM, Provost GS, Kretz PL, Dycaico MJ. 1992. Overview of the Big Blue™ in vivo mutagenesis assay: Mammalian Mutagenicity Study Group Communications. J Environ Mutagen Soc 6:74–89.

Sisk SC, Pluta L, Bond JA, Recio L. 1994. Molecular analysis of *lacI* mutants from bone marrow of $B6C3F_1$ transgenic mice following inhalation exposure of 1,3-butadiene. Carcinogenesis 15:471–477.

Skopek TR. 1995. Of mice and mutants: Target size and sensitivity. Mutat Res 331:225–228.

Tates AD, van Dam FJ, deZwart FA, van Teylingen CMM, Natarajan AT. 1994. Development of a cloning assay with high cloning efficiency to detect induction of 6-thioguanine-resistant lymphocytes in spleen of adult mice following in vivo inhalation exposure to 1,3-butadiene. Mutat Res 309:299–306.

Tates AD, Van Dam FJ, Van Teylingen CM, de Zwart FA, Zwinderman AH. 1998. Comparison of induction of *hprt* mutations by 1,3-butadiene and/or its metabolites 1,2-epoxybutene and 1,2,3,4-diepoxybutane in lymphocytes from spleen of adult male mice and rats in vivo. Mutat Res 397:21–36.

Thornton-Manning JR, Dahl AR, Bechtold WE, Griffith WC Jr, Henderson RF. 1995. Disposition of butadiene monoepoxide and butadiene diepoxide in various tissues of rats and mice following a low-level inhalation exposure to 1,3-butadiene. Carcinogenesis 16:1723–1731.

Walker VE, Gorelick NJ, Andrews JL, Craft TR, deBoer JG, Glickman BW, Skopek TR. 1996. Frequency and spectrum of ethylnitrosourea-induced mutation at the *hprt* and *lacI* loci in splenic lymphocytes of exposed *lacI* transgenic mice. Cancer Res 56:4654–4661.

1,3-Butadiene: Cancer, Mutations, and Adducts

Part IV: Molecular Dosimetry of 1,3-Butadiene

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ABSTRACT

Analysis of N7-guanine adducts derived from 1,3-butadiene (BD)* was conducted with use of liquid chromatography–mass spectrometry (LC-MS) in combination with stable isotope methods. The N7-guanine adducts were shown to undergo spontaneous depurination from DNA in vitro in both calf-thymus DNA and TK6-cell DNA. A comparison was made between BD-derived N7-guanine adduct concentrations both in liver DNA and urine of rats and mice exposed to BD. This has provided insight into the exposure of the animals to 1,2-epoxy-3-butene (BDO), 1,2,3,4-diepoxybutane (BDO_2), and 1,2-dihydroxy-3,4-epoxybutane (BDO-diol), the three oxidative metabolites of BD thought to be responsible for BD-mediated carcinogenesis.

The liver DNA of mice contained more of the two N7-guanine adducts of BDO—N7-2-hydroxy-3-butenyl-1-guanine (2HB1G) and N7-1-hydroxy-3-butenyl-2-guanine (1HB2G)—than the amounts in rats during the 10-day BD exposure and the 6 days after exposure that were monitored. An excess of 1HB2G over 2HB1G by a factor of approximately 10 in the rat liver and a factor of approximately 5 in the mouse liver was also observed. This regioselective difference was apparent during both the 10-day exposure and the 6 days after exposure. The half-lives of 2HB1G and 1HB2G were estimated as 4.3 days and 3.5 days, respectively, in the DNA of BD-exposed mice and rats. Higher amounts of 2HB1G and 1HB2G appeared in rat urine compared with mouse urine after the 10-day exposure to 1,250 ppm BD.

Analysis of liver DNA for N7-guanine adducts derived from BDO_2 revealed the presence of two diastereomeric forms of N7-(2,3,4-trihydroxybutyl)-1-guanine (THBG). One of the diastereomers [(±)-THBG] was formed by reaction of DNA with (±)-BDO_2 or BDO-diol, and the other diastereomer (*meso*-THBG) was formed by reaction of DNA with *meso*-BDO_2 or BDO-diol. There was more (±)-THBG and *meso*-THBG in liver DNA of mice compared with amounts in rats during the 10 days of BD exposure and the 6 days after exposure. A twofold excess of (±)-THBG over *meso*-THBG in rat liver was found at all of the time points monitored. After 10 days of exposure to BD, (±)-THBG in mouse liver was also present in an almost twofold excess over *meso*-THBG. At 6 days after exposure to BD, however, (±)-THBG and *meso*-THBG were present in almost equal amounts in mouse liver. Furthermore, amounts of the two THBG diastereomers in mouse liver 6 days after exposure to BD were almost fivefold greater than amounts in rat liver. The half-lives of (±)-THBG and *meso*-THBG appeared to be longer in mouse liver (4.1 days and 5.5 days, respectively) than in rat liver (3.6 days and 4.0 days, respectively). Higher amounts of (±)-THBG were excreted in rat urine compared with mouse urine.

It is noteworthy that each of the N7-guanine adducts derived from BD was present in higher concentrations in the liver DNA of mice exposed to 1,250 ppm BD than in the liver DNA of rats exposed to the same dose. Conversely, each of the adducts was present in higher concentrations in the urine of rats compared with the urine of mice after exposure to 1,250 ppm BD.

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

This Investigators' Report is Part IV of Health Effects Institute Research Report Number 92, which also includes four additional Investigators' Reports (Parts I through III and V) by different research groups, a Commentary by the Health Review Committee about Parts IV and V, a Commentary on Part I, a Commentary on Parts II and III, and an HEI Statement about the five butadiene research projects reported here. Correspondence regarding the Part IV Investigators' Report may be addressed to Dr. Ian A. Blair, Director, Center for Cancer Pharmacology, 1254 BRB II/III, 421 Curie Boulevard, University of Pennsylvania, Philadelphia, PA 19104-6160.

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INTRODUCTION

BACKGROUND

1,3-Butadiene is a colorless gas with a boiling point of –4.5°C (Merck 1998). It is used in the rubber, resins, and

plastics industries for production of poly-BD, styrene-BD, and other polymers. Butadiene is a major organic chemical with an annual production of approximately 1.6 million tons in the United States and approximately 6.6 million tons worldwide (Fajen et al. 1993).

The toxicology and epidemiology of BD have recently been reviewed in great detail (Himmelstein et al. 1997). Sources of human exposure to BD include cigarette smoke (Brunnemann et al. 1990), automobile exhaust (Neligan 1962), and industrial processes (Fajen et al. 1990). The low levels of BD (0.5 to 10 ppb) that have been detected in ambient air in urban locations are thought to arise from incomplete combustion from mobile sources (Melnick and Kohn 1995). Because BD is a volatile gas, uptake in humans occurs almost exclusively by inhalation and subsequent absorption through the respiratory tract (Himmelstein et al. 1997). Inhaled BD is carcinogenic in both rats (Owen et al. 1997) and mice (Huff et al. 1985; Melnick et al. 1990), with mice substantially more sensitive than rats. This sensitivity suggests that humans may also be at risk from exposure to this chemical.

In 1990, the Occupational Safety and Health Administration (OSHA) proposed an eight-hour time-weighted average exposure limit of 2 ppm (Himmelstein et al. 1997), a reduction from the transitional permissible exposure limit of 1,000 ppm (OSHA 1990). Further reductions in exposure limits are currently under consideration. These evaluations of exposure limits have stimulated a number of groups to develop molecular dosimetry techniques so that a safe level for BD exposure can be established.

As discussed in more detail in the introductory section on metabolism, BD is metabolized initially to a monoepoxide (BDO) and subsequently to a diepoxide (BDO_2). Both epoxides can react with bases in DNA to form DNA adducts. The exact mechanism of BD-induced carcinogenesis is not known, but the ability to quantify BD-induced DNA adducts could provide a useful dosimeter to monitor the genotoxic risk to human populations. One way to address this problem is to study BD-exposed animals to assess the concentrations of DNA adducts both in tissue samples and in urine (Shuker and Farmer 1992). A correlation between the DNA-adduct tissue concentrations with those found in urine would then provide the basis for human exposure studies that use noninvasive urinary measurements (Shuker and Farmer 1992).

METABOLISM OF BD

The metabolism of BD is extremely complex (Figure 1). Initial oxidation is mediated by cytochrome P450 2E1 (*CYP2E1*) to form BDO (Csanady et al. 1992; Duescher and Elfarra 1994; Shimada et al. 1994). There is also evidence that human myeloperoxidase can perform this oxidation (Duescher and Elfarra 1992). BDO is a substrate for glutathione *S*–transferase and epoxide hydrolase, which lead to formation of a glutathione adduct and 1,2-dihydroxy-3-butene (BD-diol), respectively. Further *CYP2E1*-mediated oxidation of BDO leads to the formation of BDO_2 (Seaton et al. 1995; Krause and Elfarra 1997). Oxidation of BD-diol by *CYP2E1* leads to the production of BDO-diol. BDO-diol can also be formed by the action of epoxide hydrolase on BDO_2. Each of these epoxide metabolites can be detoxified by the action of glutathione *S*–transferase (Sharer et al. 1991; Csanady et al. 1992) and epoxide hydrolase (Csanady et al. 1992; Cheng and Ruth 1993). In vitro studies have shown that the ratio of activation of BD to BDO compared with detoxification to glutathione adducts and BD-diol is greater (up to 12-fold) in mouse liver microsomes compared with ratios in rat and human liver microsomes (Bond et al. 1995). This provides a possible explanation for why mice may be more susceptible to BD-induced carcinogenesis than rats. (For a detailed discussion, see Himmelstein et al. 1997.)

Several groups have examined stereoselectivity (the preferential formation of one stereoisomer, which is not necessarily optically active) in the oxidation of BD and its metabolites. In a recent study with rat liver microsomes, BD was shown to be metabolized initially to three times as much (*S*)-BDO as (*R*)-BDO (Nieusma et al. 1997). Over time, however, concentration of the (*R*)-enantiomer eventually became greater than that of the (*S*)-enantiomer. In mouse liver microsomes, virtually no enantioselectivity (the preferential formation of one optically active isomer)

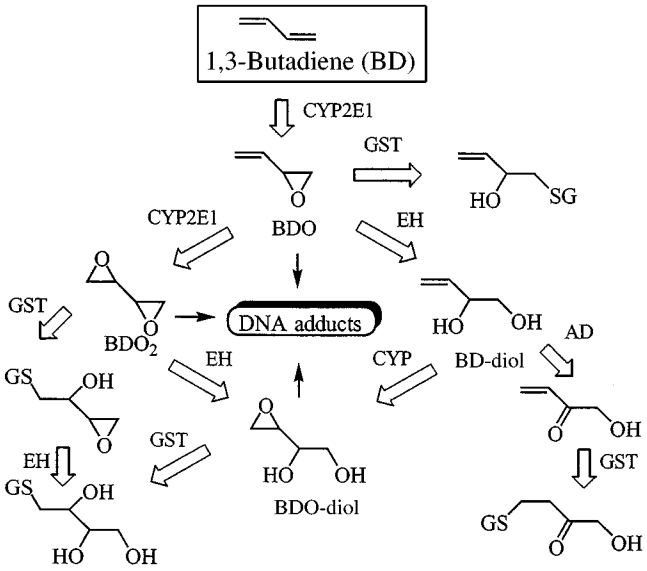

Figure 1. Metabolism of 1,3-butadiene. GST = glutathione *S*–transferase; EH = epoxide hydroxylase; and AD = alcohol dehydrogenase.

occurred in the formation of BDO (Nieusma et al. 1997). In the further oxidation of BDO with rat liver microsomes, there was a trend to more rapid formation of BDO_2 from (*R*)-BDO compared with formation from (*S*)-BDO. In contrast, with mouse liver microsomes, BDO_2 was formed from (*S*)-BDO in greater amounts than from (*R*)-BDO (Nieusma et al. 1997). In a study with recombinant human *CYP2E1*, BDO was shown to undergo metabolism to a mixture of *meso*-BDO_2 and (±)-BDO_2 (Krause and Elfarra 1997). Interestingly, *meso*-BDO_2 was more readily hydrolyzed in rat and human liver microsomes than in mouse liver microsomes (Krause and Elfarra 1997). Furthermore, hydrolysis of *meso*-BDO_2 was faster than that of (±)-BDO_2 in rat and human microsomes.

It is noteworthy that commercially available BDO_2 contains only (±)-BDO_2, and the more toxic *meso*-BDO_2 (Krause and Elfarra 1997) is totally absent. Surprisingly, none of the inhalation exposure studies have been conducted with the more toxic *meso* form of BDO_2—a major component of biologically produced BDO_2.

TOXICITY AND CARCINOGENICITY OF BD

Exposure to high concentrations of BD (that is, greater than 100,000 ppm) results in acute toxicity both to laboratory animals and to humans (Himmelstein et al. 1997). At intermediate concentrations (about 10,000 ppm), adverse reactions include irritation to the eyes and respiratory tract, together with central nervous system effects. However, exposure to the low levels of BD (< 1 ppm) encountered under modern industrial hygiene practices is not thought to cause adverse reactions or central nervous system effects (Himmelstein et al. 1997). The carcinogenic potential of long-term exposure to low levels of BD is much more controversial. Metabolic activation of BD is required before it can act as a mutagen, and so it is classified as an indirectly acting mutagen. BD and the metabolites BDO and BDO_2 are genotoxic in a number of organisms, including bacteria, yeast, and mice, as well as in mammalian cells in culture (IARC 1986). Data are insufficient to assess the in vivo carcinogenic potential of the DNA adducts formed from BDO and BDO_2, although a number of ongoing studies are designed to determine in vivo carcinogenicity.

Several in vivo studies have examined species differences in the genotoxic effects of inhalation exposure to BD. In a study conducted at the International Institute of Synthetic Rubber Producers (IISRP 1981), male and female Sprague-Dawley rats were exposed to 0, 1,000, or 8,000 ppm BD for 105 weeks. Treatment-related tumors were observed in mammary glands and in thyroid follicular cells of female rats. In male rats, testicular Leydig cell tumors were observed together with pancreatic exocrine adenomas. In contrast, chronic inhalation studies conducted in $B6C3F_1$ mice as part of the National Toxicology Program (NTP 1984) showed that BD was a multiple organ carcinogen. After exposure of mice to 625 or 1,250 ppm BD for 60 weeks, male and female mice had tumors in similar tissues. Because of the high mortality in this study, a second study was performed at lower BD concentrations (6.25 to 625 ppm) (NTP 1993). Multiple tumors were found again although the female mice were more susceptible than the male mice at these lower BD concentrations. It was apparent from these studies that $B6C3F_1$ mice are much more susceptible to BD-induced carcinogenesis than are Sprague-Dawley rats.

In other studies, exposure of $B6C3F_1$ mice to BD resulted in significant increases in the number of micronuclei and sister chromatid exchanges, but no such effects were observed in rats (Adler et al. 1994; Autio et al. 1994). These genotoxic differences are thought to reflect the species difference in carcinogenicity previously noted. When mice were exposed to BD, an increase in gene mutations at the endogenous *hprt* gene (Cochrane and Skopek 1994; Tates et al. 1994) was observed in T lymphocytes and at transgenes in the tissues of transgenic animals (Recio et al. 1992; Sisk et al. 1994). These studies demonstrated that inhaled BD causes an increased frequency of gene mutations in mice. Other studies of mutational spectra in oncogenes and tumor suppressor genes have provided additional insight into the mechanism of BD-mediated carcinogenesis in mice. Thus, G → C transversions were observed in codon 13 of K-*ras* isolated from BD-induced mouse tumors (Goodrow et al. 1990). Such data are consistent with the induction of micronuclei and sister chromatid exchanges that have been observed in BD-inhalation studies with mice.

HUMAN EPIDEMIOLOGY

Mechanistic and epidemiologic studies of BD exposure in human subjects are still very controversial: Two relatively recent reviews on the role of BD in human carcinogenesis came to quite opposite conclusions. In the first review (Melnick and Kohn 1995), mice were proposed to be a good model for human exposure to BD, and therefore humans were thought to be susceptible to BD-mediated carcinogenesis. In the second review, humans were deemed to be more like rats and therefore less susceptible to BD-mediated carcinogenesis (Bond et al. 1995). A number of epidemiologic studies suggest that there may be a causal relationship between BD exposure and excess mortality from cancers (Melnick and Kohn 1995). In the detailed study conducted by Sathiakumar and colleagues

(1998), excess mortality from leukemia was detected with increasing (estimated) occupational exposure to BD. However, workers were exposed to both styrene and BD, so it is possible that an interaction between these two chemicals was responsible for the increased incidence of leukemia (Himmelstein et al. 1997). In contrast, workers who were exposed to BD alone did not appear to show an increase in leukemia rates (Divine and Hartman 1996). Therefore, it has been concluded that BD is moderately leukemogenic, and its effect is characterized by an extended latent-induction period (Himmelstein et al. 1997). The International Agency for Research on Cancer classifies BD as a type 2A carcinogen (that is, a probable human carcinogen) (IARC 1986).

DNA ADDUCTS AND CARCINOGENESIS AFTER BD EXPOSURE

The development of cancer is the culmination of a series of molecular genetic events that induce mutations in DNA. This process can be mediated through DNA-adduct formation, which increases the probability of errors during DNA replication. Specific types and locations of DNA adducts have been linked with the mutational spectra of specific carcinogenic agents (Greenblatt et al. 1994). These DNA adducts can then mediate the activation of protooncogenes, the inactivation of tumor suppressor genes, or both (Weinberg 1989). Genetic modifications induced by DNA adducts can then initiate a series of events that causes cells to proliferate beyond their normal constraints. DNA damage in the form of modified bases or DNA adducts has been linked to modification of genes directly implicated in human cancer. Identifying and quantifying DNA adducts in tissue at various stages of neoplasia might lead to a correlation of specific mutations with specific steps in neoplastic progression. In one model of carcinogenesis, sequential steps occur in which normal cells are converted to premalignant cells, which then lead to localized tumors, invasive tumors, and finally, to metastatic lesions (Loeb 1991). The extent of malignant behavior can be the consequence of a series of genetic alterations that may be acquired through covalent modifications to DNA. These mutations can occur spontaneously or can be a result of DNA damage accumulated over a period of time. DNA adducts possess mutagenic potential and may contribute to the etiology of genetic disease and, therein, to the incidence of cancer. DNA adducts, if not repaired prior to cell replication, can induce gene mutations and putatively initiate the exposed cells, converting them to irreversibly altered preneoplastic cells (Harris 1985; Perera 1988). Once a cell is initiated, it can undergo successive mutations through promotional and progressional stages that may lead to malignancy. Therefore, the formation of DNA adducts during exposure to BD can occur continuously and may represent a critical step in the carcinogenic process (Weinstein 1988; Henderson et al. 1989).

BDO and BDO_2 are both capable of forming covalent adducts at several sites on DNA bases. A number of DNA adducts have been identified from reactions between BDO and BDO_2 molecules and relevant DNA bases or nucleosides (Citti et al. 1984; Neagu et al. 1995; Selzer and Elfarra 1996a,b; Tretyakova et al. 1997a,b). For example, a regioisomeric pair of N7-guanine adducts—2HB1G and 1HB2G—were found in BDO-treated calf-thymus DNA (Figure 2) (Citti et al. 1984; Neagu 1995; Tretyakova et al. 1997a). Similarly, calf-thymus DNA exposed to (±)-BDO_2 was shown to contain THBG (Figure 3) (Tretyakova et al. 1997b). N7-Guanine adducts of BDO have also been detected in the liver DNA of rats exposed to radiolabeled BD (Jelitto et al. 1989; Bolt and Jelitto 1996), some of which have been identified through the use of [^{32}P]-postlabeling (Koivisto et al. 1997). The absolute concentration of BDO-derived DNA adducts (as determined by [^{32}P]-postlabeling) after a 5-day, 200 ppm

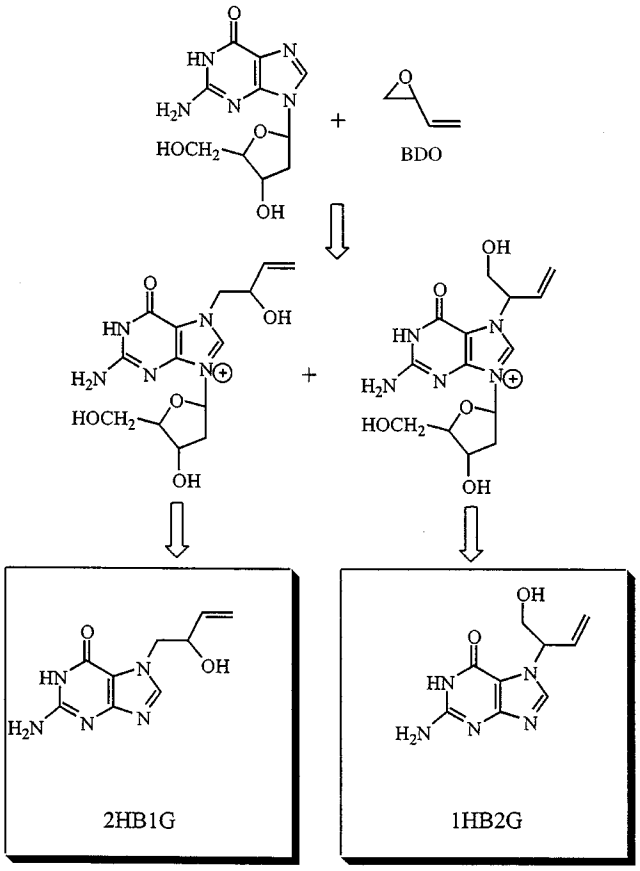

Figure 2. Formation of the N7-guanine adducts of BDO, 2HB1G and 1HB2G.

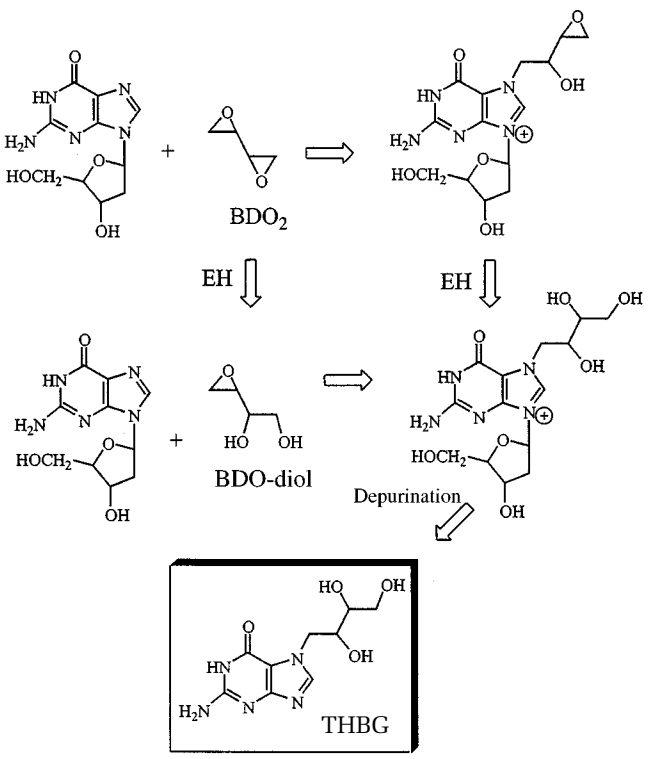

Figure 3. Formation of the N7-guanine adduct of BDO_2, THBG. EH = epoxide hydroxylase.

BD exposure was approximately 2 adducts/10^7 normal bases. When compared with 2HB1G, the excess of 1HB2G was approximately 1.5 to 1 (Koivisto et al. 1997). In addition to this stereoselectivity, there was also an observed enantioselectivity in which adducts derived from (*R*)-BDO were in 36% excess over those derived from (*S*)-BDO. This assay represents a significant contribution because it highlights the potential for both regioselective and stereoselective incorporation of BDO into DNA in vivo. However, there was a significant difference in [^{32}P]-postlabeling efficiency of 2HB1G (42%) compared with the efficiency for 1HB2G (10%). Therefore, there may be some concern with batch-to-batch variations in labeling efficiency. This will make it difficult to use the method routinely for precise and accurate quantitation of N7 adducts present in the DNA of animals exposed to BD.

A method for the analysis of BD-derived DNA adducts involving the use of stable isotope dilution LC-MS was reported recently (Tretyakova et al. 1998). In this study, rats and mice were exposed via inhalation with 1,250 ppm BD for 10 days, and liver DNA was then analyzed for DNA adducts. Methods for LC-MS should provide a more rigorous method for DNA-adduct quantitation than methods based on [^{32}P]-postlabeling. Surprisingly, no stereoselectivity was observed in the 2HB1G and 1HB2G adducts present in the DNA. However, the very short chromatographic run times employed in this analysis would make it difficult to separate the two regioisomers. Significant quantities of THBG were also observed in this study, although under the conditions employed it would not be possible to determine whether any THBG was derived from *meso*-BDO_2.

BIOMARKERS FOR EXPOSURE TO BD

The volatility of BD and the reactivity of BDO and BDO_2 make them challenging analytical targets for in vivo studies. Nevertheless, concentrations of BDO and BDO_2 in the blood and tissues of animal models after dosing with BD have been determined by the use of novel gas chromatography–mass spectrometry (GC-MS) methods (Himmelstein et al. 1994, 1995; Thornton-Manning et al. 1995). The mouse (which is the more susceptible rodent) was found to have to higher blood (Himmelstein et al. 1994) and tissue (Himmelstein et al. 1995; Thornton-Manning et al. 1995) concentrations of BDO and BDO_2 than was the rat. These data indicate that tissue samples could be employed for BD dosimetry studies conducted with animal models. For human studies, however, alternative strategies are required. Two major urinary metabolites of BDO have been identified in animals: 1,2-dihydroxy-4-(*N*-acetylcysteinyl)-butane (MI) and 1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene (MII) (Figure 4) (Sabourin et al. 1992; Bechtold et al. 1994). Species differences have been observed in the amounts of MI and MII excreted in urine. Mice produced 25% of MI compared with the amount of MII, whereas rats produced 75% of MI compared with the amount of MII. Monkeys produced MI exclusively. On the basis of these studies, urinary MI concentrations have been proposed as a biomarker for BD exposure in human populations (Bechtold et al. 1994).

Protein adducts have been utilized extensively as biomarkers for compounds that react intrinsically with proteins or undergo activation in vivo to reactive substances (Skipper and Tannenbaum 1990). BDO and BDO_2, the two identified reactive metabolites of BD, can react with proteins such as hemoglobin that circulate in the blood. The long life span of erythrocytes, 4 months, has made hemoglobin adducts attractive biomarkers for exposure to alkylating agents such as BD (Osterman-Golkar et al. 1976, 1993). The steady-state level of hemoglobin adducts during chronic exposure to BD has been employed as a measure of total BD exposure during the life span of the erythrocytes (van Sittert and van Vliet 1994; Osterman-Golkar 1996). The dose-response for formation of hemoglobin's *N*-terminal

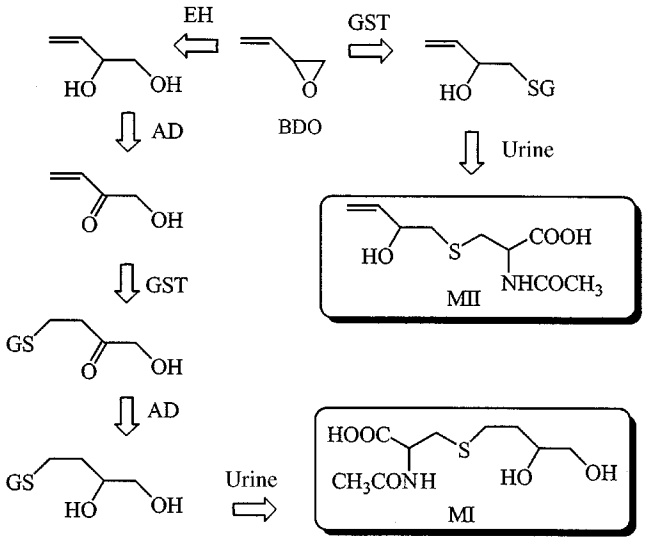

Figure 4. Formation of the urinary metabolites of BDO, MI and MII. EH = epoxide hydroxylase; GST = glutathione *S*–transferase; and AD = alcohol dehydrogenase.

valine adduct with BDO (that is, 2-hydroxy-3-butenyl-valine) and the stability of this adduct have been established in BD-exposed rats and mice (Albrecht et al. 1993; Osterman-Golkar 1993). As noted previously, the blood and tissue concentrations of BDO were greater in BD-exposed mice than in BD-exposed rats (Himmelstein et al. 1994, 1995; Thornton-Manning et al. 1995). In keeping with this observation, the *N*-valine adduct 2-hydroxy-3-butenyl-valine was present in concentrations that were 3 to 8 times higher in BD-exposed mice than in BD-exposed rats (Albrecht et al. 1993; Osterman-Golkar 1993). A study of human industrial workers exposed to BD (at the level of 11 ± 18.6 mg/m^3) revealed that higher concentrations of 2-hydroxy-3-butenyl-valine were present in their hemoglobin (namely, 0.16 pmol/g) compared with the concentration in hemoglobin of nonexposed workers (0.05 pmol/g) (Osterman-Golkar 1996). This study suggests that the *N*-terminal valine adduct of hemoglobin can be employed to monitor low-level BD exposure.

The quantitation and identification of DNA adducts in urine or tissue samples as molecular dosimeters require sophisticated analytical methods because the adducts are normally present in such low concentrations (Santella 1988). Techniques employed to analyze these DNA adducts include: [^{32}P]-postlabeling (Reddy and Randerath 1986; Reddy et al. 1989), immunochemical methods (Santella 1988; Santella et al. 1990; Wild 1990; Degan et al. 1991), fluorescence spectroscopy (Weston and Bowman 1991), high-pressure liquid chromatography (HPLC) with electrochemical detection (Goda and Marnett 1991), gas chromatography–electron ionization–mass spectrometry of trimethylsilyl derivatives (Dizdaroglu 1986; Shigenaga 1989; Bonfanti et al. 1990; Djuric et al. 1991), gas chromatography–electron capture, negative chemical ionization mass spectrometry after derivatization with pentafluorobenzyl bromide (Chaudhary et al. 1994; Giese 1997), liquid chromatography–thermospray–mass spectrometry (Jajoo et al. 1992), liquid chromatography–continuous-flow fast-atom bombardment (Wolf and Vouros 1994), and liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Chaudhary et al. 1995). Mass spectrometric techniques have high sensitivity and are highly specific because only ions derived from the analyte of interest are monitored. Unfortunately, the expense of setting up this method and the expertise required to operate the instrumentation have tended to limit its use for quantitative analyses of DNA adducts that may be present in biological samples.

The development of LC-MS based upon the atmospheric pressure ionization techniques of electrospray ionization (ESI) and ionspray (nebulizer-assisted ESI) have had a profound effect upon our ability to identify analytes of interest when only trace amounts of material are available (Blair 1993). These techniques are extremely robust and powerful when employed in combination with MS/MS and collision-induced dissociation (CID) on triple quadrupole instruments. For MS/MS studies, an ion of one particular mass is allowed to pass through the first analyzer region (Q1) of the triple quadrupole into the second (radio frequency only) analyzer region (Q2), where it is allowed to collide with an inert gas. This collision results in the formation of product ions. The product ions are then separated in the third analyzer region (Q3) and transmitted to the detector (Fenselau 1992). Tandem mass spectroscopy is a powerful structural tool because it permits the initial characterization of endogenous compounds (based on the product ion profile) from trace amounts of material.

For quantitative studies, selected reaction monitoring (SRM) is preferred because higher sensitivity can be attained. In this mode, the protonated molecular ion (MH^+) is allowed to pass through Q1, and CID is performed in Q2. An intense product ion is separated in Q3 and allowed to pass through to the detector. This results in high sensitivity together with extremely high specificity. The specificity arises because the analyte has to have the correct HPLC retention time, the correct molecular ion, and the correct product ion (Blair 1993). The LC-MS technique is rapidly becoming the method of choice for quantitative analysis of DNA adducts in the picomole range for three reasons: no derivatization is required, LC is efficient, and MS/MS provides high specificity. The present study

was initiated in order to develop LC-MS/MS methods for analysis of N7-guanine adducts in the tissue and urine of animals exposed to BD.

In summary, three different types of biomarkers can be considered for use in molecular dosimetry studies of human BD exposure: a urinary BDO metabolite (MI), a BDO hemoglobin adduct (2-hydroxy-3-butenyl-valine), and the N7-guanine adducts of BDO and BDO_2 (2HB1G, 1HB2G, and THBG). The purpose of the present study was to determine whether a relation exists between tissue and urine concentrations of 2HB1G, 1HB2G, and THBG and the known sensitivity of relevant animal models to BD-induced carcinogenesis. If such a correlation were found, then the urinary assay could be employed in future human dosimetry studies of BD exposure.

SPECIFIC AIMS

SPECIFIC AIM 1: TO CHARACTERIZE DNA ADDUCTS FORMED FROM THE IN VIVO METABOLITES OF BD

Previous studies established that BDO and BDO_2 were major in vivo metabolites of BD. Therefore, in order to complete the first specific aim, it was necessary to synthesize authentic N7-guanine adducts of BDO and BDO_2 and to develop a method based on LC-MS/MS for the analysis of these compounds. The LC-MS/MS technique was then used for the characterization of BDO and BDO_2 adducts in calf-thymus DNA. Heavy isotope–labeled, standard 2′-deoxynucleosides were synthesized for preparation of isotope-labeled N7-guanine adducts of BDO and BDO_2 for use in quantitative studies. Initial quantitative studies were performed using in vitro systems, calf-thymus DNA, and TK6-cell DNA. These studies were conducted in order to provide information on the stability of the adducts and on the relative amounts of adducts that were incorporated into DNA.

SPECIFIC AIM 2: TO CHARACTERIZE AND QUANTITATE DNA ADDUCTS DERIVED FROM THE IN VIVO METABOLITES OF BD IN TISSUES AND URINE OF MICE AND RATS

The LC-MS/MS method developed as part of specific aim 1 was validated for the analysis of N7-guanine adducts of BDO and BDO_2 in tissue samples and in urine. During the validation procedures, the lower limit of quantitation for each of the DNA adducts was defined. A major objective of specific aim 2 was to determine the relative amounts of the N7-guanine adducts of BDO and BDO_2 in the tissues and urine of BD-exposed animals. This would then lay the foundation for human dosimetry studies. It was of particular interest to determine whether any of the guanine adducts arose from *meso*-BDO_2 as this would provide insight into the in vivo formation of BDO_2.

METHODS AND STUDY DESIGN

CHEMICALS

Adenine, racemic BDO, racemic BDO_2, 2′-deoxyguanosine, guanine, guanosine, phosphorus oxychloride, and trifluoroacetic acid (TFA) were purchased from Aldrich Chemical (St. Louis, MO). The compounds BDO and BDO_2 are mutagens and required handling with particular care. The compound [^{13}C]formamide was obtained from Isotec (Miamisburg, OH). Two guanosine adducts, N7-(2-hydroxy-3-buten-1-yl)guanosine (1 mg) and N7-(1-hydroxy-3-buten-2-yl)guanosine (1 mg), were gifts of Dr. Elfarra (Selzer and Elfarra 1996b). [$^{15}N_5$]Guanine was prepared by acid hydrolysis of [$^{15}N_5$]guanosine (Cambridge Isotopes, Andover, MA). Hydrochloric acid (HCl) and Optima water were purchased from Fisher Scientific (Pittsburgh, PA). Calf-thymus DNA was purchased from Sigma Chemical (Milwaukee, WI). Solid-phase extraction (SPE) C_{18} cartridges (3 mL, 500 mg) were obtained from Varian (Harbor City, CA). Methanol, ethanol, isopropanol, chloroform, nitric acid, ammonium acetate (NH_4OAc), potassium hydroxide (KOH), acetic acid (AcOH), and CoStar nylon centrifuge filters (0.22 μm) were purchased from VWR Scientific Products (Bridgeport, NJ). Bond Elut C_{18} SPE columns (6 mL, 500 mg) and phenylboronic acid (PBA) bonded silica SPE columns (6 mL, 500 mg) and C_8 strong cation exchange Certify columns (3 mL, 50 mg) were purchased from Varian (Harbor City, CA). Proteinase K, lysis buffer, phenol/chloroform/water solution, sodium acetate (2 M), and precipitette filters (25 mm), specifically prepared for the Genepure 341 DNA extractor, were purchased from Applied Biosystems (Foster City, CA). Nitrogen (N_2), helium, and argon gases were supplied by BOC Gases (Bellmawr, NJ).

INSTRUMENTATION AND EQUIPMENT

All HPLC-ultraviolet testing was conducted using a Hitachi L-6200 series liquid chromatography device (Hitachi, San Jose, CA) interfaced to a Hitachi L-4200 UV detector. Nuclear magnetic resonance (NMR) spectra were obtained with a 300 MHz or 400 MHz instrument (Bruker, Billerica, MA). Ultraviolet spectra were recorded with a Beckman 550 instrument (Beckman, Palo Alto, CA). Centrifuges (Juoan, Winchester, VA, or Beckman, Columbia, MD)

were used to pellet DNA or whole cells. A Genepure 341 nucleic acid purification system (Applied Biosystems, Foster City, CA) was employed for the TK6-cell and liver-DNA extractions. Tissue homogenization was performed using a Polytron (model PCU11) homogenizer from Kinematica AG (Littau, Switzerland). A RapidTrace SPE workstation (Zymark, Hopkinton, MA) was used for automated SPEs. When necessary, samples were evaporated to dryness under N_2 gas at 35°C in an N-Evap device (Organomation, South Berlin, MA). LC-MS was performed using a Waters 2690 Alliance LC (Millipore, MA) interfaced to a Finnigan MAT TSQ7000 triple quadrupole mass spectrometer (Finnigan, San Jose, CA). Postacquisition data analysis was performed using Finnigan LC Quan Version 1.2 RC1 software (Finnigan, San Jose, CA). Preparative HPLC was performed on a Beckman C_{18} analytical column (250 × 10 mm i.d., 5 µm, Alltech Associates, Deerfield, IL), a YMC-AQ ODS analytical column (150 × 2.0 mm i.d., 3 µm; YMC, Wilmington, NC), or a semipreparative column (250 × 10 mm i.d., 5 µm; Alltech Associates). YMC-AQ ODS columns (250 × 2.0 mm i.d., 5 µm; or 250 × 2.0 mm i.d., 3 µm; or 150 × 2.0 mm i.d., 3 µm) were used for LC-MS studies.

ANIMALS

Animal studies were conducted by Dr.Vernon Walker and Dr. Leslie Recio at the Chemical Industry Institute for Toxicology. The rodents, F344 rats and $B6C3F_1$ mice, were exposed to 1,250 ppm BD for 6 hours/day, 5 days/week over 2 weeks. Urine samples (total 24) were collected from four of the rats and four of the mice daily for 3 days after exposure to BD. Five mice and five rats were killed after 1 day, 5 days, or 10 days of exposure. Five mice and five rats were also killed at 1 day, 3 days, or 6 days after exposure to BD. All of the animals were killed by exsanguination. Organs were then collected, packed in foil, frozen, and shipped at −80°C to the analysis site. They were stored at −80°C until analyzed.

CHEMICAL SYNTHESES

BDO-Guanine Adducts 2HB1G and 1HB2G

Two guanosine adducts, N7-(2-hydroxy-3-buten-1-yl)guanosine (1 mg) and N7-(1-hydroxy-3-buten-2-yl)guanosine (1 mg), were acid hydrolyzed with 2 M HCl (0.5 mL) to form 2HB1G and 1HB2G, respectively. Acid hydrolysis was performed for 3 hours at 80°C. The solution was cooled and KOH (2 M, 0.5 mL) was added to neutralize the hydrolysis reaction before isolation and analysis. Preparative LC was conducted using a Beckman C_{18} column (250 × 4.6 mm i.d.; 5 µm) maintained at room temperature with a flow rate of 1 mL/min. Solvent A was 0.1% TFA in water/acetonitrile solution (99:1), and solvent B was 0.1% TFA in water/acetonitrile solution (90:10). A linear gradient elution was performed as follows: 0 minute, 0% B; 10 minutes, 0% B; 18 minutes, 100% B; 22 minutes, 100% B; 25 minutes, 0% B. 2HB1G had a retention time of 18.6 minutes, and 1HB2G had a retention time of 19.5 minutes.

Proton NMR (^{1}H-NMR) profiles of 1HB2G and 2HB1G were essentially identical. The ^{1}H-NMR profile of 1HB2G (deuterated-dimethylsulfoxide [D_6-DMSO]; 300 MHz) was as follows: δ 3.73 [dd, 1H, C_{10}-H′; J_{gem} = 11.1 Hz; J_{vic} = 4.7 Hz], 3.92 [dd, 1H, C_{10}-H′′; J_{gem} = 11.1 Hz; J_{vic} = 7.9 Hz], 5.12 [d, 1H, C_{13}-H′; J_{trans} = 17.0 Hz], 5.19 [m, 1H, C_{11}-H], 5.22 [d, 1H, C_{13}-H′′; J_{cis} = 10.4 Hz], 6.19 [ddd, 1H, C_{12}-H; J_{trans} = 17.0 Hz; J_{cis} = 10.4 Hz; J_{vic} = 7.0 Hz], 6.40 [s, 1H, C_2-NH_2], 8.10 [s, 1H, C_8-H], 11.00 [br s, 1H, N_1-H]. Authentic samples of 1HB2G and 2HB1G were dissolved in water, and their concentrations were determined by UV absorbance at λ_{max} 250 nm in 0.1 N HCl (ε 9970) (Citti et al. 1984).

[2,4,5,6,8-$^{13}C_5$]Adenine

A mixture of [^{13}C]formamide (0.5 g, 11.1 mmol) and phosphorus oxychloride (3.4 g, 22.2 mmol) was placed in a 15-mL glass tube (Ace, Aldrich, Milwaukee, WI), sealed with a type B plug, and heated at 120°C for 15 hours. The glass tube was cooled to room temperature and then to 0°C in an ice bath. The stopper was slowly opened, and water (20 mL) was added to the tube. This solution was vortex mixed with charcoal (1 g) and filtered. The brown solution was neutralized to pH 7 with ammonium hydroxide (NH_4OH) at 0°C. This solution was lyophilized, and the residue was purified by silica-gel column chromatography with methylene chloride and methanol (10%) as the solvent. The combined fractions were evaporated to give [$^{13}C_5$]adenine as a white solid (0.151 g, 48%), with ^{1}H-NMR (D_6-DMSO; 300 MHz) of δ 12.8 [bs, 1H, exchangeable with deuterated water (D_2O)], 8.07 [dm, 2H], 7.07 [s, 2H, exchangeable with D_2O]. The ^{13}C-NMR profile (D_6-DMSO; 50.32 MHz) was 155.20 [d; J = 71.2 Hz], 152.04 [s], 151.17 [d; J = 71.2 Hz], 139.28 [s], 117.50 [m].

[$^{13}C_4$,^{15}N]Guanosine

4,5,6,8-[$^{13}C_4$]-9-[^{15}N]Guanosine was prepared as described by Yeola and colleagues (1994). The [$^{13}C_4$,^{15}N]guanine was converted to the nucleoside [$^{13}C_4$,^{15}N]guanosine using a modification of the procedure reported by Chapeau and Marnett (1991): Uridine was substituted for thymidine during the enzymatic reaction. The [$^{13}C_4$,^{15}N]guanosine (6 mg) was purified using a semipreparative C_{18} column (250 × 10 mm i.d.; 5 µm) at a flow rate of 2 mL/min. Solvent A was water, and solvent B was methanol/water (9:1 v/v). An isocratic system consisting of 20% solvent B was

used to purify the stable isotope-labeled guanosine (retention time 8.5 minutes).

[$^{13}C_4$,^{15}N]2HB1G and [$^{13}C_4$,^{15}N]1HB2G Internal Standards

Syntheses of the BDO-N7 adducts of [$^{13}C_4$,^{15}N]guanosine were completed using the method of Selzer and Elfarra (1996b). The adducts N7-(2-hydroxy-3-buten-1-yl)[$^{13}C_4$,^{15}N]guanosine (2 mg) and N7-(1-hydroxy-3-buten-2-yl)[$^{13}C_4$,^{15}N]guanosine (2 mg) were hydrolyzed to the corresponding guanine adducts using 2 M HCl (6 mL). Acid hydrolysis was performed for 3 hours at 80°C. The resulting solution was cooled and neutralized with 2 M KOH (6 mL). The guanine adducts [$^{13}C_4$,^{15}N]2HB1G and [$^{13}C_4$,^{15}N]1HB2G were unequivocally characterized by their chromatographic and mass spectral properties: They had the identical LC retention times as the unlabeled standards. In addition, their mass spectra showed an increase of 5 Da in protonated molecular ions and major product ions compared with the spectra of the unlabeled standards.

(±)-BDO_2–Guanine Adduct (±)-THBG

The adduct (±)-THBG was synthesized by modification of the method reported by Citti and colleagues (1984) for the preparation of 2HB1G and 1HB2G. Briefly, (±)-BDO_2 (40 µL, 44.5 mg, 0.52 mmol) was added to guanosine (25.5 mg, 0.090 mmol) in AcOH (1 mL). The AcOH solution was heated at 60°C for 4 hours. A mixture of acetone/diethyl ether (5 mL, 1:4) was added to the solution. The resulting precipitate was isolated by centrifugation (5°C, 3,000 rpm, 5 minutes) and was hydrolyzed by heating at 80°C for 2.5 hours in 1 N HCl (1 mL). The (±)-THBG was purified using a semipreparative C_{18} column (250 × 10 mm i.d.; 10 µm). Solvent A was 5 mM aqueous NH_4OAc containing 0.1% TFA, and solvent B was 5 mM NH_4OAc containing 0.1% TFA in methanol. A linear gradient elution was performed as follows: 0 minute, 5% B; 2 minutes, 5% B; 5 minutes, 15% B; 20 minutes, 17% B; 25 minutes, 50% B; 30 minutes, 80% B; 35 minutes, 95% B; 40 minutes, 5% B, at a flow rate of 1.5 mL/min. The retention time of (±)-THBG under these conditions was 16.0 minutes. (±)-THBG was isolated as the trifluoroacetate salt. The ^{1}H-NMR (D_6-DMSO; 400 MHz) profile was as follows; δ: 3.2 ~ 3.5 [m, 3H, C(4′)-H2 and C(2′ or 3′)-H], 3.81 [brs, 1H, C(3′ or 2′)-H], 4.11 [dd, 1H, C(1′)-H; J_{gem} = 13.6 Hz; J_{vic} = 8.8 Hz], 4.28 [dd, 1H, C(1′)-H; J_{gem} = 13.6 Hz; J_{vic} = 4.0 Hz], 4.48 [t,1H, C(4′)-OH; J_{vic} = 5.4 Hz], 4.66 and 4.67 [q-like each d, each 1H, C(2′)-OH and C(3′)-OH; each J = 3.0 Hz], 6.09 [s, 2H, NH_2], 7.2 [brs, 1H, NH], 7.79 [s, 1H, C(8)-H]. In addition, the isolated (±)-THBG produced other characteristic data: MS/MS with a mass-to-charge ratio (m/z) of m/z 256 to 152 (due to loss of N7-alkyl side chain) and UV absorbance (in 0.1 N HCl) of 271 nm (shoulder, ε 6800), 251 nm (maximum, ε 9926), 229 nm (minimum, ε 5511).

(±)-BDO_2–[$^{13}C_4$,^{15}N]Guanine Adduct (±)-[$^{13}C_4$,^{15}N]THBG

The adduct (±)-4,5,6,8-[$^{13}C_4$]-9-[$^{15}N_1$]THBG was synthesized from 4,5,6,8-[$^{13}C_4$]-9-[$^{15}N_1$]guanosine using the same method described above for the unlabeled compound.

meso-BDO_2–N7-Guanine Adduct *meso*-THBG

A mixture of *meso*-BDO_2 and (±)-BDO_2 was synthesized by a modification of the method reported by Sangaiah and colleagues (1997). Dimethyldioxirane (DMDO) in acetone (10 mL) was added to a solution of BDO (200 µL, 174 mg, 2.48 mmol) in acetone (1 mL) at –80°C. The solution was maintained at –15°C for 2.5 hours, and then N_2 gas was bubbled through the solution at 20°C for 60 minutes to remove excess DMDO and BDO. The residue, which contained a mixture of *meso*-BDO_2 and (±)-BDO_2 (40 µL), was added to guanosine (25.0 mg, 0.08 mmol) in AcOH (1 mL). The AcOH solution was heated to 60°C for 22 hours, then concentrated to approximately 0.5 mL under N_2 gas. Acetonitrile/diethyl ether (6 mL, 1:5) was added and the resulting precipitate was isolated by centrifugation (5°C, 3,000 rpm, 10 minutes). The precipitate, which contained a mixture of guanosine-BDO_2 adducts, was hydrolyzed by heating at 80°C for 2.5 hours in 1N HCl (1 mL).

Preliminary purification of THBG regioisomers was performed by reverse-phase preparative HPLC using a semipreparative C_{18} column (250 mm × 4.6 mm i.d.; 5 µm) at a mobile-phase flow rate of 1.0 mL/min. Solvents A and B were the same as described for the preparation of (±)-THBG. A linear gradient was used: 0 minute, 5% B; 2 minutes, 5% B; 5 minutes, 15% B; 20 minutes, 17% B; 22 minutes, 95% B; 30 minutes, 95% B; 32 minutes, 5% B; 40 minutes, 5% B. The fraction, which eluted between 6 minutes and 8 minutes, was concentrated under N_2 gas at 50°C. The residue was dissolved in 1 mL H_2O containing 10 µL of 1N HCl. The less polar *meso*-THBG was then separated from (±)-THBG by reverse-phase preparative HPLC using an analytical YMC AQ-ODS column (150 mm × 4.6 mm i.d.; 3 µm). The solvents and flow rate were the same as those used for the semipreparative column. A linear gradient was employed: 0 minute, 2% B; 2 minutes, 2% B; 10 minutes, 12% B; 11 minutes, 2% B; 15 minutes, 2% B. Under these conditions, the *meso*-THBG (which was isolated as a trifluoroacetate salt) had a retention time of 9.9 minutes and a ^{1}H-NMR (D_6-DMSO/D_2O; 400 MHz) profile as follows; δ: 3.3–3.5 [m, 2H, CH_2-4′b and H-3′], 3.55 [m, 1H, CH_2-4′b], 3.70 [m, 1H, H-2′], 4.05 [dd, 1H, CH_2-1′a; J_{gem} = 14.0 Hz; J_{vic} = 8.0 Hz], 4.48

[dd, 1H, CH_2-1′b; J_{gem} = 14.0 Hz; J_{vic} = 2.0 Hz], 7.80 [s, 1H, H-8]. Other data were obtained with MS/MS of *m/z* 256 to 152 (due to loss of N7-alkyl side chain) and UV absorbance (in 0.1 N HCl) of λ 271 nm (shoulder), 251 nm (maximum), 229 nm (minimum).

Acetylation of (±)-THBG and *meso*-THBG in Biological Samples

The residue from purified rat urine or evaporated tissue hydrolysate was dissolved into pyridine (300 µL) and acetic anhydride (180 µL), and then heated at 60°C overnight. The reaction mixture was dried down under a N_2 stream, redissolved in water (2 mL), and then applied to a C_{18} SPE column. The column was rinsed with water (1 mL) and acetonitrile/water (1 mL; 1:9), and the fraction containing the tetraacetate derivative was eluted with acetonitrile/water (2 mL; 9:1). After evaporating the sample derivative to dryness under a N_2 stream, the residue was redissolved in 200 µL of mobile phase solvent A. It was filtered through a 0.2 µm nylon membrane by centrifugation at 1,400 rpm for 1 minute and analyzed by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) techniques.

PREPARATION OF BIOLOGICAL SAMPLES

BDO-Guanine Adducts in Calf-Thymus DNA

BDO (30 µL, 25.8 mg, 369 µmol) was dissolved in methanol (270 µL), and then 10 µL of the solution (0.86 mg, 12.2 µmol BDO) was added to a series of tubes containing calf-thymus DNA (100 µg, 0.3 mol of DNA bases) in water (0.5 mL). The solutions were vortex mixed and incubated at 37°C. Tubes were removed in duplicate at 0.25 hour, 0.5 hour, 1 hour, 3 hours, 6 hours, and 12 hours. Four tubes were incubated for 24 hours. Excess BDO was evaporated under N_2 gas at room temperature, and the residual solution was treated with 3 M aqueous NH_4OAc (50 µL) and ice-cold 95% ethanol (1.5 mL). Precipitated DNA was pelleted by centrifugation (20 minutes, 3,000 rpm), and the ethanol supernatant was saved for analysis of spontaneously depurinated 2HB1G and 1HB2G. Two of the 24-hour samples of BDO-treated DNA were redissolved in 1 mL of water and heated at 37°C for 24 hours. The DNA was isolated as described above, and the ethanol supernatant was removed for LC-MS analysis. This procedure was repeated for two additional 24-hour periods, to produce four ethanol supernatant samples (24, 48, 72, and 96 hours) together with the DNA that had been incubated for 96 hours. The samples were stored at room temperature for up to 2 hours until they were analyzed.

BDO-Guanine Adducts in TK6-Cell DNA

TK6 (human lymphoblastoid) cell incubations were performed by Dr. Leslie Recio at the Chemical Industry Institute of Toxicology. Four replicate experiments were performed in which TK6 cells (4×10^5 cells/mL) suspended in 50 mL of culture medium were exposed to 400 µM BDO for 24 hours. Immediately after exposure, the four samples were transferred to 50-mL tubes, and TK6 cells were collected by centrifugation. The supernatants were transferred to large conical tubes and stored at −20°C. Cell pellets from two of the incubations were transferred to 1.5-mL Eppendorf tubes and centrifuged briefly at 15,000 rpm. The residual supernatant was removed, and the cells were frozen at −20°C. Cell pellets from the other two incubations were resuspended in fresh medium and allowed to grow for an additional 72 hours. Cells and medium were then collected as described in the previous section. Samples were shipped at −80°C and stored at −80°C until analyzed. For analysis of DNA adducts, 2×10^7 cells were transferred to 15-mL polypropylene tubes, allowed to thaw on ice for 1 hour, and then rinsed three times with 5 mL phosphate-buffered saline (PBS). They were mixed with a pipette and centrifuged at 3,000 rpm at 4°C for 15 minutes. The pellet was then resuspended in PBS (4 mL).

Extraction of DNA from TK6 Cells

The TK6-cell pellet in PBS (4 mL) was transferred to the 30-mL glass extraction vessel of the Genepure 341 automated DNA extractor. The lysis buffer was added and heated to 60°C for 10 minutes; proteinase K digestion was then performed for 1 hour at 60°C. Two phenol/chloroform extractions and one chloroform extraction were performed. Aqueous NH_4OAc (3 M) was added, followed by 95% ethanol. Each vessel was carefully examined for the presence of DNA. If all vessels had visible DNA, it was collected on the 25-mm precipitette filters. The DNA was allowed to dry in the hood for 30 minutes, and then it was dissolved in 1 mL water for DNA quantitation. The samples were vortex mixed until the DNA dissolved (time range, 30 minutes to 18 hours). In rare cases, the automated DNA extractor did not give rise to DNA strands. In these cases, the DNA was precipitated by centrifugation at 4,000 rpm for 30 minutes at 5°C. The solution was decanted, and DNA samples were transferred to 2-mL polypropylene centrifuge tubes. The DNA was rinsed three times with 1 mL ice-cold ethanol. After each addition of ethanol, the sample was vortex mixed and centrifuged at 14,000 rpm for 10 minutes at 4°C. The DNA was air dried in the hood for 30 minutes and then dissolved in 1 mL water and DNA quantified as described below. DNA was then reprecipitated by the addition of 3 M aqueous

NH_4OAc (100 µL) and ethanol (3 mL). The tube was centrifuged at 3,000 rpm for 30 minutes at 4°C, and the supernatant decanted. The DNA was allowed to air dry in the hood for 30 minutes. Samples were stored at room temperature for a maximum of 2 hours before they were analyzed.

Quantitation of DNA

A 10-µL aliquot of DNA solution (approximately 500 µg/mL) was transferred to a quartz cuvette with a 1-cm path length. Water (90 µL) was added, the solution was mixed with a disposable pipette and allowed to stand for 5 minutes. The UV absorbance (*A*) was recorded at 260 nm and 280 nm. If the ratio of *A*260/*A*280 was between 1.7 and 1.9, the DNA concentration was determined from the absorbance at 260 nm (A_{260} = 50 µg DNA). This allowed calculation of the total number of DNA bases from the equivalence relation that 300 µg DNA equaled 1 mol of DNA base. If the purity of the DNA was inadequate, a second extraction was performed using the Genepure DNA extractor.

Comparison of Hydrochloric Acid and Formic Acid for Hydrolysis of Calf-Thymus DNA Containing BDO_2-Guanine Adducts

BDO_2 (6.1 µL, 6.9 mg, 80 µmol) was added to calf-thymus DNA (100 mg) in water (200 µL). This solution was incubated at 37°C for 135 minutes, then evaporated under N_2 for 15 minutes to remove excess BDO_2. The sample was divided into two portions, and DNA was precipitated with 3 M NH_4OAc (50 mL) and ice-cold ethanol (1.5 mL). After centrifugation of the two samples, the DNA was separated. The ethanol supernatants were evaporated to dryness. One of the residues from the ethanol supernatant and its corresponding DNA sample were treated with 200 µL 2M HCl for 3 hours at 80°C. The other residue from the ethanol supernatant and its corresponding DNA sample were treated with 88% formic acid (30 µL) in water (170 µL) and heated for 3 hours at 80°C. The samples were cooled to room temperature, neutralized with 4 M KOH, and diluted to 2 mL with water. Extractions were performed using a Zymark automated RapidTrace system as described previously.

Preparation of Samples for LC-MS Analysis of 2HB1G and 1HB2G in Rat and Mouse Urine

Tubes containing human urine (500 µL) were spiked with the following amounts of 1HB2G and 2HB1G: 0 ng, 0.068 ng, 0.137 ng, 0.273 ng, 2.73 ng, 8.22 ng, 13.7 ng, or 27.3 ng. Duplicate samples from human urine pools containing appropriate amounts of 2HB1G and 1HB2G were included with each analysis for lower, middle, and higher concentration quality control (LQC, MQC, and HQC). Rat urine samples (500 µL) were transferred by pipette into glass tubes. Mouse urine samples (100 µL) were also transferred by pipette into glass tubes but they were diluted to 500 µL with water. The internal standards [$^{13}C_4$,^{15}N]2HB1G (2.7 ng) and [$^{13}C_4$,^{15}N]1HB2G (2.7 ng) were added to each urine sample, each urine standard, and each quality control (QC) sample. Additional samples (to which no internal standards were added) included a water blank and a sample containing only human urine (500 µL). Samples were extracted using a Zymark RapidTrace system as described for the validation assay.

Preparation of Samples for Analysis of (±)-THBG in Rat and Mouse Urine

Tubes containing human urine (500 µL) were diluted with water to 2 mL and spiked with the following amounts of (±)-THBG: 0 ng, 0.276 ng, 0.691 ng, 1.38 ng, 2.76 ng, 6.91 ng, 13.8 ng, or 37.6 ng in order to construct a standard curve. Duplicate LQC, MQC, and HQC samples from human urine pools containing appropriate amounts of (±)-THBG were included with each analysis. Rat urine samples (500 µL) were transferred by pipette into glass tubes and diluted to 2 mL with water. Mouse urine samples (100 µL) were also transferred by pipette into glass tubes and diluted to 2 mL with water. The internal standard (±)-[$^{13}C_4$,^{15}N]THBG (17.0 ng) was added to each urine sample, each urine standard, and each QC sample. Additional samples (to which no internal standards were added) included a water blank and a sample containing only human urine (500 µL). Samples were extracted as described for the validation assay.

Extraction of Tissue Samples from Rats and Mice

Rat or mouse liver (500 mg) was weighed and placed into a 15-mL polypropylene screw-cap tube, which was then placed on ice. Ice-cold PBS (5 mL) was added to each liver tissue sample. The samples were homogenized on ice using a Polytron homogenizer and then filtered through a piece of cotton gauze (2 inches × 2 inches) into a clean polypropylene 15-mL tube. The homogenizer was rinsed with 5 mL of clean PBS, and this was added to the previous filtrate. The tissue homogenate samples were centrifuged at 3,000 rpm for 20 minutes at 4°C, and the DNA was extracted using the Genepure 341 automated DNA extractor as described for TK6-cell DNA. The isolated DNA was dissolved in water (1 mL) and quantified using spectrophotometry. Samples were stored at room temperature for up to a week in readiness for LC-MS analysis.

Preparation of Rat- and Mouse-Liver DNA and Quality Control Samples for LC-MS Analysis

[$^{13}C_4$,^{15}N]2HB1G (1.4 ng), [$^{13}C_4$,^{15}N]1HB2G (1.4 ng), and (±)-[$^{13}C_4$,^{15}N]THBG (17 ng) internal standards were added to an aliquot of each DNA solution (250 µL) isolated from the rat or mouse liver. Tubes containing calf-thymus DNA (100 µg) in water (250 µL) were spiked with 1HB2G and 2HB1G (0 pg, 13.6 pg, 68.3 pg, 136.0 pg, 191.0 pg, 273.0 pg, 1,360.0 pg, 4,100.0 pg, or 6,830.0 pg) and (±)-THBG and *meso*-THBG (0 pg, 69.0 pg, 138 pg, 276.0 pg, 690.0 pg, 1,380.0 pg, 2,760.0 pg, 6,900.0 pg, or 13,800.0 pg). Duplicate LQC, MQC, and HQC samples from pools containing calf-thymus DNA (100 µg) in water (250 µL) were included with each analysis. The internal standards [$^{13}C_4$,^{15}N]2HB1G (1.4 ng), [$^{13}C_4$,^{15}N]1HB2G (1.4 ng), and (±)-[$^{13}C_4$,^{15}N]THBG (17 ng) were added to the standard solutions and to the QC samples. Additional samples (to which no internal standards were added) included a water blank and a sample containing only calf-thymus DNA (100 µg) in water (250 µL). DNA samples, standards, and QC samples were treated as described for the validation procedure. Samples were stored at room temperature for up to a week in readiness for LC-MS analysis.

LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY METHODS

Mass Spectrometry

Electrospray ionization (ESI) was performed in the positive mode using N_2 both as sheath (pressure, 80 psi) and auxiliary (30 psi) gas in order to assist with nebulization. A potential of 4.5 kV was applied to the ESI needle. The metal capillary was maintained at 205°C in order to provide optimum desolvation of ions generated by ESI. The skimmer pump pressure was held at 0.847 torr. Full scanning analyses were performed in the range of *m/z* 50 to *m/z* 500. CID was performed using argon as the collision gas at a pressure of 0.0025 torr at −24 ± 2 eV of energy. Full-scan product ion spectra were obtained by selecting MH^+ in Q1 and scanning Q3 in the range *m/z* 50 to *m/z* 250. For SRM experiments, tube lens and capillary voltages were optimized for maximum response using appropriate standards. The other mass spectrometer parameters were optimized to obtain maximum sensitivity without sacrificing unit resolution. For the compounds 2HB1G and 1HB2G, SRM analyses were performed with MH^+ ions *m/z* 222 (2HB1G and 1HB2G) and *m/z* 227 ([$^{13}C_4$,$^{15}N_1$]2HB1G and [$^{13}C_4$,$^{15}N_1$]-1HB2G) selected in Q1. Q3 was monitored for the product ions *m/z* 152 (2HB1G and 1HB2G) and *m/z* 157 ([$^{13}C_4$,$^{15}N_1$]-2HB1G, and [$^{13}C_4$,$^{15}N_1$]1HB2G), which resulted from the loss of the N7-alkyl side chains. For (±)-THBG and *meso*-THBG, SRM analyses were performed with MH^+ ions *m/z* 256 [(±)-THBG and *meso*-THBG] and *m/z* 261 [(±)-[$^{13}C_4$,$^{15}N_1$]-THBG] selected in Q1. Product ions *m/z* 152 [(±)-THBG, *meso*-THBG] and *m/z* 157 [(±)-[$^{13}C_4$,$^{15}N_1$]THBG], which also resulted from the loss of N7-alkyl side chains, were monitored in Q3.

Validation of LC-MS Assay for 2HB1G and 1HB2G in Calf-Thymus DNA and TK6-Cell DNA

Tubes containing calf-thymus DNA (100 µg) in water (0.5 mL) were spiked with the following amounts of 1HB2G and 2HB1G: 0 pg, 6.8 pg, 13.6 pg, 27.3 pg, 68.2 pg, 136.0 pg, 205.0 pg, or 273.0 pg. Quality control samples from pools containing appropriate amounts of 1HB2G and 2HB1G together with calf-thymus DNA (100 µg) in water (0.5 mL) were used to obtain measures of precision and accuracy. Six QC samples were used for each of the four concentration levels: the lower limit of quantitation (LLQ; 6.8 pg), LQC (27.3 pg), MQC (109 pg), and HQC (175 pg). The internal standards, [$^{13}C_4$,^{15}N]1HB2G (2.7 ng) and [$^{13}C_4$,^{15}N]2HB1G (2.7 ng), were added to the standard solutions and to the QC samples. Additional samples (to which no internal standards were added) included a water blank and a sample containing only calf-thymus DNA (100 µg) in water (0.5 mL). Analysis of these samples allowed any interfering peaks from reagents or matrix to be identified.

The samples were hydrolyzed with 2 M HCl (0.2 mL) at 80°C for 3 hours. The hydrolysates were cooled and neutralized with 2 M KOH (0.2 mL) and then diluted to 2 mL with water. Extractions were performed using a Zymark automated RapidTrace system. The SPE C_{18} (3 mL/500 mg) columns were conditioned with chloroform, followed by acetonitrile, methanol, and water (9 mL of each). The hydrolyzed standards (2 mL) and QC samples (2 mL) were applied to SPE columns and washed with 2% acetonitrile (5 mL). DNA adducts were eluted from the SPE columns with methanol/water (5 mL, 9:1). The SPE eluant was evaporated to dryness under N_2 at 35°C. The residue was dissolved in 200 µL of water, sonicated for 10 minutes, vortex mixed, centrifuged for 5 minutes at 2,000 rpm, and then filtered through a CoStar filter. The filtrates were transferred to vials and stored at room temperature for up to a week before LC-MS analysis was conducted. On two separate days, standard curve samples, together with six replicate samples of the LQC, MQC, and HQC solutions, were prepared and stored at room temperature for up to a week in readiness for LC-MS analysis.

Preparation of Calf-Thymus DNA, TK6-Cell DNA, TK6-Cell Supernatant, Ethanol Supernatants, Standards, and Quality Control Samples for LC-MS Analysis of 2HB1G and 1HB2G

The [$^{13}C_4$,^{15}N]2HB1G (2.7 ng) and [$^{13}C_4$,^{15}N]1HB2G (2.7 ng) internal standards were added to the ethanol supernatants, TK6-cell supernatants (2 mL), and DNA samples. The ethanol supernatants were evaporated under N_2 at 35°C, and the residues were dissolved in 2 mL of water. Tubes containing calf-thymus DNA (100 μg) in water (0.5 mL) were spiked with the following amounts of 1HB2G and 2HB1G: 0 pg, 6.8 pg, 13.6 pg, 27.3 pg, 68.2 pg, 136.0 pg, 205.0 pg, or 273.0 pg. Duplicate LQC, MQC, and HQC samples from pools containing calf-thymus DNA (100 μg) in water (0.5 mL) were included with each analysis. The internal standards [$^{13}C_4$,^{15}N]2HB1G (2.7 ng) and [$^{13}C_4$,^{15}N]1HB2G (2.7 ng) were added to the standard solutions and to the QC samples. Additional samples (to which no internal standards were added) included a water blank and a sample containing only calf-thymus DNA (100 g) in water (0.5 mL). DNA samples, standards, and QC samples were hydrolyzed with 2 M HCl (0.2 mL) at 80°C for 3 hours. The hydrolysates were cooled and neutralized with 2 M KOH (0.2 mL), followed by dilution to 2 mL with water. The SPE C_{18} (3 mL/500 mg) columns were conditioned with chloroform, followed by acetonitrile, methanol, and water (9 mL of each). The DNA hydrolysates (2 mL), ethanol extracts (2 mL), TK6-cell supernatants (2 mL), hydrolyzed standards (2 mL), and hydrolyzed QC samples (2 mL) were then extracted using a Zymark automated RapidTrace system as described in the validation procedure. The CoStar filtrates were stored at room temperature for up to a week before LC-MS analysis was conducted. Adducts were identified in a separate experiment, in which no internal standard was added, by comparison of sample chromatographic and mass spectral characteristics with those of authentic standards.

LC-MS Analysis of 2HB1G and 1HB2G in Standards, Quality Control Samples, Calf-Thymus DNA, TK6-Cell DNA, Cell Supernatants, and Ethanol Supernatants

A portion of the CoStar filtrate (50 μL) was analyzed by LC-MS at a mobile-phase flow rate of 200 μL/min using a YMC AQ ODS column (250 × 2.0 mm i.d.; 5 μm). Solvent A was water/TFA (100:0.01) containing 5 mM NH_4OAc, and solvent B was methanol/water/TFA (9:1:0.01) containing 5 mM NH_4OAc. Isocratic elution was performed with a mobile phase consisting of 18% B. Retention times were 13.5 minutes for 2HB1G and 14.2 minutes for 1HB2G.

Quantitation of 2HB1G and 1HB2G in Standards, Quality Control Samples, Calf-Thymus DNA, TK6-Cell DNA, Cell Supernatants, and Ethanol Supernatants

Calibration curves were obtained by a weighted least-squares (1/x) linear-regression analysis of peak height ratios *m/z* 222–152 to *m/z* 227–157 against the amount of 2HB1G or 1HB2G in each tube. Equations of the calibration curves were then used to calculate concentrations of 2HB1G and 1HB2G in the QC and analyte samples from the peak height ratios of *m/z* 222–152 to *m/z* 227–157 (for the internal standard).

Quantitation of Normal Bases in DNA

Each DNA hydrolysate sample was spiked with [$^{13}C_5$]adenine (1 μg) and [$^{15}N_5$]guanine (1 μg) and then diluted to 100 μL. The amounts of purine bases were determined from four calibration standards containing a mixture of guanine and adenine standards (50, 100, 250, or 500 ng/sample). LC-MS was then performed on 10 μL of sample using a YMC AQ ODS column (250 × 2.0 mm; 3 μm) at a flow rate of 200 μL/min. Solvent A was aqueous 5 mM NH_4OAc containing 0.1% AcOH, and solvent B was methanolic 5 mM NH_4OAc containing 0.1% AcOH. Isocratic elution was performed using a mobile phase of 35% solvent B. SRM was performed on the reaction $[MH^+] \rightarrow [MH^+–NH_3]$ of purine bases and their respective heavy isotope–labeled internal standards, *m/z* 152 to 135 (guanine), *m/z* 157 to 140 ([$^{15}N_5$]guanine), *m/z* 136 to 119 (adenine), and *m/z* 141 to 124 ([$^{13}C_5$]adenine). Calibration curves were obtained by a weighted least-squares ($1/x^2$) linear-regression analysis of peak height ratios (*m/z* 152–135 to *m/z* 157–140 for guanine; *m/z* 136–119 to *m/z* 141–124 for adenine) against the amount of guanine or adenine in each tube. Equations of the calibration curves were then used to calculate concentrations of purine bases in the QC and analyte samples from the peak height ratios of *m/z* 152–135 to *m/z* 157–140 (guanine) and *m/z* 136–119 to *m/z* 141–124 (adenine). Concentrations of normal DNA bases were then calculated from the guanine and adenine content.

Time Course for Formation of BDO_2-Guanine Adducts in Calf-Thymus DNA

BDO_2 (8 μL, 9.4 mg, 109 μmol) was dissolved in methanol (1 mL). Portions of this methanolic BDO_2 solution (8 μL) containing 75 μg (872 nmol) of BDO_2 were added to a series of tubes containing calf-thymus DNA (100 μg, 0.33 μmol DNA bases) in water (0.2 mL). The solutions were vortex mixed and incubated at 37°C. Tubes were removed in duplicate at 0.25 hour, 0.5 hour, 1 hour, and 2 hours. Excess BDO_2 was evaporated under N_2 at room temperature, and the residual solution was treated with

3 M aqueous NH_4OAc (50 µL) and ice-cold 95% ethanol (1.5 mL). Precipitated DNA was pelleted by centrifugation (20 minutes, 3,000 rpm), and the ethanol supernatant was saved for analysis of spontaneously depurinated guanine adducts. The supernatant and the corresponding DNA were spiked with (±)-[$^{13}C_4$,^{15}N]THBG internal standard (17 ng) and treated with 200 µL 2M HCl for 3 hours at 80°C. The samples were cooled to room temperature, neutralized with 4 M KOH, and diluted to 2 mL with water. Extraction and purification of DNA adducts were performed using a Zymark automated RapidTrace system. The SPE C_{18} (3 mL/500 mg) columns were conditioned with chloroform, followed by acetonitrile, methanol, and water (9 mL of each). The DNA hydrolysates (2 mL), ethanol extracts (2 mL), standards, and QC samples were applied to SPE columns and washed with 2% acetonitrile (5 mL). THBG was eluted from the SPE columns with methanol/water (5 mL, 9:1). The SPE eluant was evaporated to dryness under N_2 at 35°C. The residue was dissolved in 200 µL of water, sonicated for 10 minutes, vortex mixed, centrifuged for 5 minutes at 2,000 rpm, and then filtered through a CoStar filter. The filtrates were transferred to vials and stored at room temperature for up to a week before LC-MS analysis was conducted.

Preparation of Standards and Quality Control Samples for Analysis of (±)-THBG in Calf-Thymus DNA

Tubes containing hydrolyzed calf-thymus DNA (100 µg) in water (1.0 mL) were spiked with the following amounts of (±)-THBG: 0 pg, 27.6 pg, 69.0 pg, 138.0 pg, 276.0 pg, 690.0 pg, 1,380.0 pg, 2,760.0 pg, 6,900.0 pg, or 13,800 pg. The internal standard (±)-[$^{13}C_4$,^{15}N]THBG (17.0 ng) was added to the standard solutions. Additional samples (to which no internal standards were added) included a water blank and a sample containing only hydrolyzed calf-thymus DNA (100 µg) in water (1.0 mL). DNA samples were hydrolyzed with 2 M HCl (0.2 mL) at 80°C for 3 hours. The hydrolysates were extracted with a Zymark automated RapidTrace system as described for the calf-thymus DNA validation procedure.

Analysis of (±)-THBG in Calf-Thymus DNA

The CoStar filtrates (80 µL) were analyzed by LC-MS at a mobile-phase flow rate of 200 µL/min using a YMC AQ ODS column (250 × 2.0 mm i.d.; 5 µm). Solvent A was water/AcOH (99.9:0.1) containing 5 mM NH_4OAc, and solvent B was methanol/AcOH (99.9:0.1) containing 5 mM NH_4OAc. A linear gradient elution was performed as follows: 0 minute, 0% B; 4 minutes, 0% B; 22 minutes, 90% B; 24 minutes, 90% B; 25 minutes, 0% B; 45 minutes, 0% B. The retention time of (±)-THBG under these conditions was 14.23 minutes.

Quantitation of (±)-THBG in Calf-Thymus DNA and in Ethanol Supernatants

Calibration curves were obtained by a weighted least-squares (1/x) linear-regression analysis of peak height ratios *m/z* 256–152 to *m/z* 261–157 against the amount of (±)-THBG in each tube. Equations of the calibration curves were then used to calculate the amounts of (±)-THBG in the QC and analyte samples from the peak height ratios of *m/z* 256–152 to *m/z* 261–157.

Validation of LC-MS Assay for 2HB1G and 1HB2G in Urine

Tubes containing human urine (500 µL) were spiked with the following amounts of 1HB2G and 2HB1G: 0 ng, 0.068 ng, 0.137 ng, 0.273 ng, 2.73 ng, 8.22 ng, 13.7 ng, or 27.3 ng in order to construct a standard curve. QC samples from pools of human urine containing appropriate amounts of 2HB1G and 1HB2G were used to obtain measures of precision and accuracy. Six samples each of the LLQ (0.136 ng/mL), the LQC (0.54 ng/mL), the MQC (4.36 ng/mL), and the HQC (38.2 ng/mL) were used. The internal standards [$^{13}C_4$,^{15}N]2HB1G (2.7 ng) and [$^{13}C_4$,^{15}N]1HB2G (2.7 ng) were added to each standard and QC sample. Additional samples (to which no internal standards were added) included a water blank and a sample containing only human urine. Samples were extracted using an automated Zymark RapidTrace system. The SPE Certify columns were conditioned with methanol (3 mL) and water (3 mL). The samples were loaded on the columns at a flow rate of 1.0 mL/min. The columns were rinsed with 3 mL 0.1 M AcOH and 9 mL water at a flow rate of 1.0 mL/min. The Certify cartridges were dried with 40 psi N_2 gas for 1 minute and rinsed with 9 mL methanol at a flow rate of 2 mL/min.

The compounds 2HB1G and 1HB2G were eluted with ethyl acetate/isopropanol/methanol (7:2:1) containing 2% concentrated NH_4OH at a flow rate of 2 mL/min. The samples were evaporated using an N-Evap at 40°C. They were then reconstituted in water (200 µL), sonicated for 20 minutes, vortex mixed for 30 seconds, and centrifuged at 2,000 rpm for 5 minutes at 10°C. The resulting solutions were filtered through CoStar 0.22-µm nylon centrifuge tubes at 14,000 rpm for 2 minutes. The samples were stored at room temperature for up to a week before they were analyzed by LC-MS. On two separate days, standard curve samples together with six replicate samples each of the LQC, MQC, and HQC solutions were prepared and

stored at room temperature for up to a week in readiness for LC-MS analysis.

Analysis of 2HB1G and 1HB2G in Rat and Mouse Urine

A portion of the SPE extract (50 µL) was analyzed by LC-MS at a mobile-phase flow rate of 200 µL/min using a YMC AQ ODS column (150 × 2.0 mm i.d.; 3 µm). Isocratic elution was performed with a mobile phase consisting of water/methanol (82:18) containing 5 mM NH_4OAc and 0.01% TFA. The retention time was 7.3 minutes for 2HB1G and 8.1 minutes for 1HB2G.

Quantitation of 2HB1G and 1HB2G in Rat and Mouse Urine

Quantitation was performed as described for the calf-thymus DNA samples.

Validation of LC-MS Assay for (±)-THBG in Urine

Tubes containing human urine (1.0 mL) were diluted with water to 2 mL and spiked with the following amounts of (±)-THBG: 0 ng, 0.276 ng, 0.691 ng, 1.38 ng, 2.76 ng, 6.91 ng, 13.8 ng, or 37.6 ng in order to construct a standard curve. QC samples from pools of human urine containing appropriate amounts of (±)-THBG were used to obtain measures of precision and accuracy. Six samples each of the LLQ (0.138 ng/mL), the LQC (0.345 ng/mL), the MQC (1.38 ng/mL), and the HQC (6.9 ng/mL) were analyzed. The internal standard (±)-[$^{13}C_4$,^{15}N]THBG (17.0 ng) was added to each sample. Additional samples (to which no internal standards were added) included a water blank and a sample containing only human urine.

The C_{18} SPE columns (6 mL/500 mg) were conditioned with chloroform (3 mL), methanol (3 mL), and water (3 mL). Samples were applied to the SPE columns and allowed to load by gravity. Each SPE column was dried by suction and then eluted with water/acetonitrile (5 mL, 1:9). After adjusting the pH of the eluates to 8.8 with 2.5 M NH_4OAc buffer (pH 8.8, 0.5 mL), the eluates were applied to PBA columns and allowed to load by gravity. The PBA columns were washed with 0.25 M NH_4OAc buffer (pH 8.8, 3 mL), and the DNA adducts were eluted with aqueous 0.1 M HCl (5 mL). After adjusting the pH to the range of 6.5 to 7.0 with 1 N KOH (about 130 µL), each fraction was applied to a second C_{18} column by gravity. Columns were dried by suction, and DNA adducts were eluted with water/acetonitrile (2 mL, 1:9). The eluates were evaporated to dryness in an N-Evap. The residues were redissolved in 200 µL of water/AcOH (99.9:0.1) containing 5 mM NH_4OAc and filtered through a 0.22-µm CoStar filter by centrifugation at 1,400 rpm for 1 minute.

Samples were stored at room temperature for up to a week until analysis. On three separate days, standard curve samples together with six replicate samples each of the LQC, MQC, and HQC solutions were prepared and stored at room temperature for up to a week in readiness for LC-MS analysis.

Analysis of (±)-THBG in Rat and Mouse Urine

The CoStar filtrates (50 µL) were analyzed by LC-MS at a mobile-phase flow rate of 200 µL/min using a YMC AQ ODS column (250 × 2.0 mm i.d.; 3 µm). Solvent A was water/methanol/AcOH (98:2:0.1) containing 5 mM NH_4OAc, and solvent B was methanol/AcOH (99.9:0.1) containing 5 mM NH_4OAc. A linear gradient elution was performed: 0 minute, 0% B; 2 minutes, 0% B; 15 minutes, 50% B; 20 minutes, 95% B; 25 minutes, 0% B; 45 minutes, 0% B. The retention time of (±)-THBG was 13.7 minutes.

Quantitation of (±)-THBG in Rat and Mouse Urine

Quantitation was performed as described previously for the calf-thymus DNA samples.

Separation of (±)-THBG from *meso*-THBG in Mouse Urine

LC-MS was conducted at a mobile-phase flow rate of 200 µL/min using a YMC AQ ODS column (250 × 2.0 mm i.d.; 3 µm). Solvent A was water/methanol/AcOH (98:2:0.1) containing 5 mM NH_4OAc, and solvent B was methanol/AcOH (99.9:0.1) containing 5 mM NH_4OAc. A linear gradient elution was performed as follows: 0 minute, 0% B; 2 minutes, 0% B; 25 minutes, 50% B; 25.1 minutes, 50% B; 30 minutes, 95% B; 35 minutes, 95% B; 40 minutes, 0% B; 55 minutes, 0% B. The retention times of (±)-THBG and *meso*-THBG were 15.60 minutes and 16.05 minutes, respectively.

Validation of LC-MS Assays for 2HB1G, 1HB2G, and (±)-THBG in Rat- and Mouse-Liver DNA Samples

Tubes containing calf-thymus DNA (100 µg) in water (250 µL) were spiked with the following amounts of 1HB2G and 2HB1G: 0 pg, 13.6 pg, 68.3 pg, 136.0 pg, 191.0 pg, 273.0 pg, 1,360.0 pg, 4,100.0 pg, or 6,830.0 pg and the following amounts of (±)-THBG: 0 pg, 69.0 pg, 138.0 pg, 276.0 pg, 690.0 pg, 1,380.0 pg, 2,760.0 pg, 6,900.0 pg, or 13,800.0 pg. Five QC samples from pools containing appropriate amounts of 2HB1G, 1HB2G, and (±)-THBG together with calf-thymus DNA (100 µg) in water (250 µL) were used to obtain measures of precision and accuracy. Five samples each of the LLQ, the LQC, the MQC, and the HQC preparations were used. For 2HB1G and 1HB2G the LQC was 27.3 pg, the MQC was 109.0 pg,

and the HQC was 190.0 pg. For (±)-THBG and *meso*-THBG, the LLQ was 69.0 pg, the LQC was 138.0 pg, the MQC was 1,380.0 pg, and the HQC was 13,800.0 pg. The internal standards [$^{13}C_4$,^{15}N]2HB1G (1.4 ng), [$^{13}C_4$,^{15}N]1HB2G (1.4 ng), and (±)-[$^{13}C_4$,^{15}N]THBG (17 ng) were added to the standard solutions and to the QC samples. Additional samples (to which no internal standards were added) included a water blank and a sample containing only calf-thymus DNA (100 µg) in water (250 µL). Standards and QC samples were hydrolyzed with 88% formic acid (20 µL) at 80°C for 1.5 hours. The hydrolysates were cooled and neutralized with concentrated NH_4OH (14 µL). Acetonitrile (0.5 mL) was added to each sample. The samples were evaporated to dryness at 35°C in an N-Evap, reconstituted in 250 mL of water, and filtered through CoStar filters at 14,000 rpm for 2 minutes. The filtrates were transferred to vials and stored at room temperature for up to a week in readiness for LC-MS analysis. On two separate days, standard curve samples, together with five replicate samples of the LQC, MQC, and HQC solutions, were prepared and ready for LC-MS analysis.

Analysis of 2HB1G, 1HB2G, (±)-THBG, and *meso*-THBG in Rat- and Mouse-Liver DNA Samples

The CoStar filtrates (50 µL) from the DNA hydrolyses were analyzed by LC-MS at a mobile-phase flow rate of 150 µL/min using a YMC AQ ODS column (250 × 2.0 mm i.d.; 3 µm). Solvent A was water/methanol/AcOH (98:2:0.1) containing 5 mM NH_4OAc, and solvent B was methanol/AcOH (99.9:0.1) containing 5 mM NH_4OAc. A linear gradient elution was performed: 0 minute, 0% B; 2 minutes, 0% B; 22 minutes, 60% B; 23 minutes, 95% B; 28 minutes, 95% B; 35 minutes, 0% B; 45 minutes, 0% B. The following retention times were observed: (±)-THBG, 15.3 minutes; *meso*-THBG, 16.1 minutes; 2HB1G, 20.5 minutes; and 1HB2G, 22.0 minutes.

Quantitation of 2HB1G, 1HB2G, (±)-THBG, and *meso*-THBG in Rat- and Mouse-Liver DNA Samples

For 2HB1G and 1HB2G, calibration curves were obtained by a weighted least-squares (1/x) linear-regression analysis of peak height ratios *m/z* 222–152 to *m/z* 227–157 against the amount of 2HB1G or 1HB2G present. Equations of the calibration curves were then used to calculate concentrations of 2HB1G and 1HB2G in the QC and analyte samples from the peak height ratios of *m/z* 222–152 to *m/z* 227–157. For (±)-THBG and *meso*-THBG, calibration curves were obtained by a weighted least-squares (1/x) linear-regression analysis of peak height ratios *m/z* 256–152 to *m/z* 261–157 against the amount of (±)-THBG or *meso*-THBG present. Equations of the calibration curves were then used to calculate concentrations of (±)-THBG in the QC and analyte samples from the peak height ratios of *m/z* 256–152 to *m/z* 261–157.

Data Analysis, Accuracy, and Precision

All LC-MS/MS data were processed by Finnigan LC Quan 1.2 software. This allowed automated determination of regression lines to be performed and analyte amounts to be determined by an automated procedure using the calculated regression lines. The accuracy of the assays was evaluated by calculating the mean percent deviation from the expected value. Intra- and interassay (interday) precision were evaluated by calculating the within error expressed as the percentage relative to the standard deviation.

STATISTICS

An analysis of variance (ANOVA) appropriate for a two-factorial experimental design was performed using the general linear model procedure of SAS (Cary, NC) software. If examination of the second-order interactions (animal model by time) revealed a significant effect ($p < 0.20$), this precluded an examination of the main effects. In these cases, an examination of simple effects of animal model stratified by time was performed. For each time point, a Student *t* test of independent samples was conducted. A significant difference was defined by $p < 0.05$.

RESULTS

LC-MS ANALYSIS OF AUTHENTIC 2HB1G AND 1HB2G

LC-MS analysis of 2HB1G and 1HB2G revealed intense MH^+ ions for each analyte at *m/z* 222. Both CID and MS/MS analysis resulted in the formation of an intense ion at *m/z* 152 for both 2HB1G (Figure 5A) and 1HB2G (data not shown). These product ions arose from loss of the N7-alkyl side chains. LC-MS analysis of [$^{13}C_4$,$^{15}N_1$]2HB1G and [$^{13}C_4$,$^{15}N_1$]1HB2G also revealed that there were intense MH^+ ions for each analyte. However, the MH^+ ion was shifted by 5 Da to *m/z* 227 compared with the *m/z* value for the unlabeled guanine adducts. The contribution of unlabeled material at *m/z* 222 was less than 0.1% of the total. CID of the MH^+ ions and MS/MS analysis resulted in formation of an intense ion at *m/z* 157 for both [$^{13}C_4$,$^{15}N_1$]2HB1G (Figure 5B) and [$^{13}C_4$,$^{15}N_1$]1HB2G (data not shown). These product ions also arose from loss of the N7-alkyl side chains.

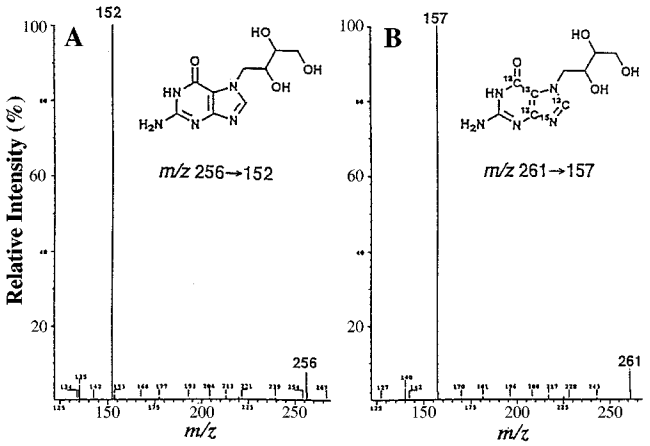

Figure 5. Product ion spectra for 2HB1G. Spectrum derived from CID and MS/MS analysis of (A) *m/z* 222 from 2HB1G and (B) *m/z* 227 from [$^{13}C_4$,$^{15}N_1$]2HB1G.

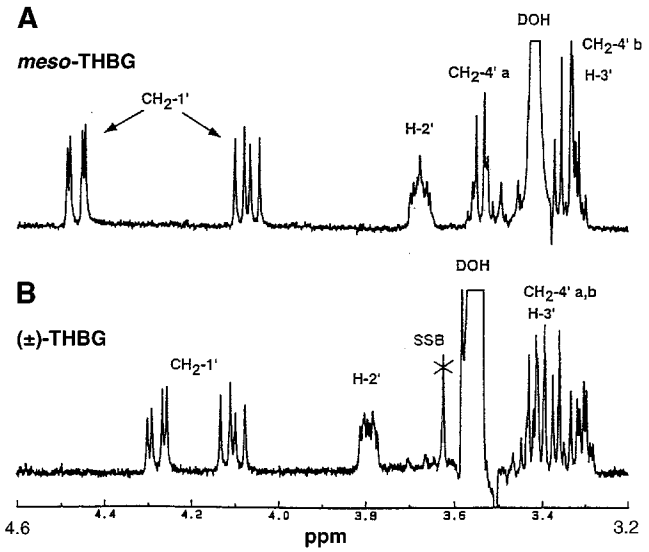

Figure 6. ^{1}H-NMR spectra of *meso*-THBG (A) and (±)-THBG (B). Both lie in the region of δ 3.2 to 4.6 ppm.

SYNTHESIS OF AUTHENTIC (±)-THBG AND *meso*-THBG

Authentic (±)-THBG was prepared from (±)-BDO_2 and characterized by ^{1}H-NMR spectroscopy. In D_6-DMSO, the CH-8 methine appeared as a singlet at 7.8 ppm, the proton at N^1-H as a broad signal at 7.2 ppm, the two protons on the exocyclic amino group at C-2 as a singlet at 6.09 ppm, the two protons on hydroxyl groups attached at C-2′ and C-3′ as a complex signal at 4.66 ppm, and the proton on the hydroxyl group at C-4′ as a triplet at 4.48 ppm. The signals for the two protons at CH_2-1′ appeared as doublets of doublets at two different chemical shifts. Thus, CH_2-1′a appeared at 4.28 ppm and CH_2-1′b at 4.11 ppm (Figure 6). The methine proton at CH-2′ was observed as a complex signal at 3.81 ppm. Signals from the methine proton at CH-3′ and the methylene protons (a,b) at CH_2-4′ appeared as a complex signal between 3.2 and 3.5 ppm. These latter signals were only revealed when D_2O was added to the DMSO. Authentic *meso*-THBG was isolated from the reaction between guanosine and a mixture of (±)-BDO_2 and *meso*-BDO_2. Although *meso*-THBG and (±)-THBG had similar LC retention times and UV spectra, the ^{1}H-NMR spectrum of *meso*-THBG was quite distinct from that of (±)-THBG in the region 3.2 ppm to 4.6 ppm (Figure 6).

Heteronuclear multiple quantum correlation analysis of *meso*-THBG confirmed the assignments of the protons in the ^{1}H-NMR spectrum (Figure 7). Thus, the methylene carbon that gave rise to a signal at 50 ppm was attached to the two protons at 4.48 ppm and 4.09 ppm. From the chemical shift, this carbon was assigned as C-1′ (attached to N and CHOH), and so the signals at 4.48 ppm and 4.09 ppm were derived from the CH_2′-1a,b protons. The other methylene carbon, which gave rise to a signal at 63 ppm, was attached to the two protons at 3.55 ppm and 3.2 to 3.5 ppm. This carbon was assigned as C-4′ because it was more deshielded (that is, attached to OH and CHOH) than the other methylene carbon, observed at 50 ppm. Therefore, the signal at 3.55 ppm and a component of the signal at 3.2 to 3.5 ppm were derived from the two CH_2′-4a,b protons. The methine carbon, which gave rise to a signal at 70 ppm, was attached to a proton at 3.68 ppm. This carbon was assigned as C-2′ based on its chemical

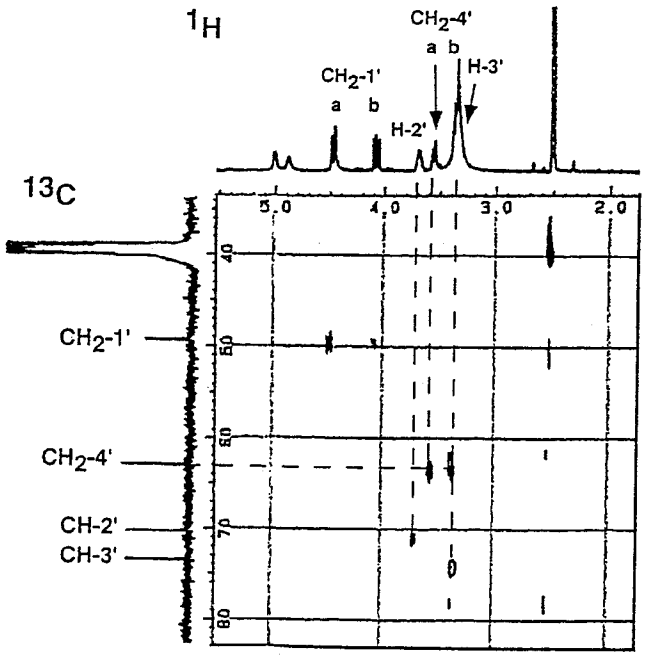

Figure 7. Heteronuclear multiple quantum correlation analysis of *meso*-THBG.

shift (attached to CH_2N, OH, and CHOH), and so the signal at 3.68 ppm was derived from the CH-2′ proton. The second methine carbon, which gave rise to a signal at 73.5 ppm, was attached to a proton at 3.2 to 3.5 ppm. This carbon was assigned as C-3′ because it was more de-shielded (that is, attached to CHOH, OH, and CH_2OH) than the other methine carbon at 70 ppm. Therefore, a component of the signal at 3.2 to 3.5 ppm was assigned to the CH-3′ proton.

LC-MS ANALYSIS OF (±)-THBG AND *meso*-THBG

LC-MS analysis of (±)-THBG and *meso*-THBG revealed intense MH^+ ions for each analyte at *m/z* 256. CID and MS/MS analysis resulted in the formation of an intense ion at *m/z* 152 (protonated guanine) for both (±)-THBG (Figure 8A) and *meso*-THBG (data not shown). These product ions arose from loss of the N7-alkyl side chains. LC-MS analysis of (±)-[$^{13}C_4$,$^{15}N_1$]THBG revealed that there was also an intense MH^+ ion for this analyte. The MH^+ ion was shifted by 5 Da to *m/z* 261 compared to the *m/z* value for the unlabeled THBG. The contribution of unlabeled material at *m/z* 256 was less than 0.1% of the total CID of the MH^+ ion and MS/MS analysis resulted in the formation of an intense ion at *m/z* 157 (Figure 8B). This product ion also arose from loss of the N7-alkyl side chain.

VALIDATION OF LC-MS ASSAY FOR 2HB1G AND 1HB2G IN CALF-THYMUS DNA AND TK6-CELL DNA

Calibration standards for 2HB1G and 1HB2G were analyzed in the range of 6.8 pg to 273 pg. A typical calibration curve for 2HB1G had a regression line of y = 0.005 + 0.009x (r^2 = 0.998); for 1HB2G, a typical regression line was y = 0.002 + 0.001x (r^2 = 0.999). The back-calculated calibration standards were within ±10% of the expected values. A reagent blank, a control (unexposed) calf-thymus DNA sample, a control (unexposed) TK6-cell DNA sample, a control calf-thymus DNA sample with internal standard, and a control TK6-cell DNA sample with internal standard confirmed that there were no interfering substances from the reagents or DNA. Typical LC-SRM-MS chromatograms for BDO-exposed calf-thymus DNA and TK6-cell DNA samples are shown in Figures 9 and 10, respectively. Intraassay precision for the 2HB1G QC samples ranged from 0.02% to 7.7%; deviation from the nominal concentration (that is, accuracy) ranged from 102% to 107.7%. Intraassay precision for the 1HB2G QC samples ranged from 0.02% to 3.4%; deviation

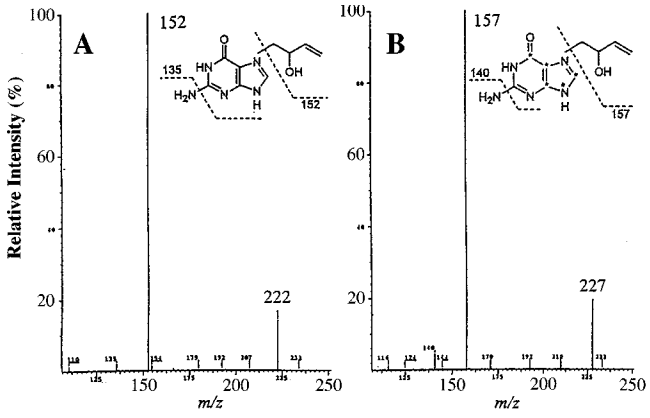

Figure 8. Product ion spectra for (±)-THBG. Spectrum derived from CID and MS/MS analysis of (A) *m/z* 256 from (±)-THBG and (B) *m/z* 261 from (±)-[$^{13}C_4$,$^{15}N_1$]THBG.

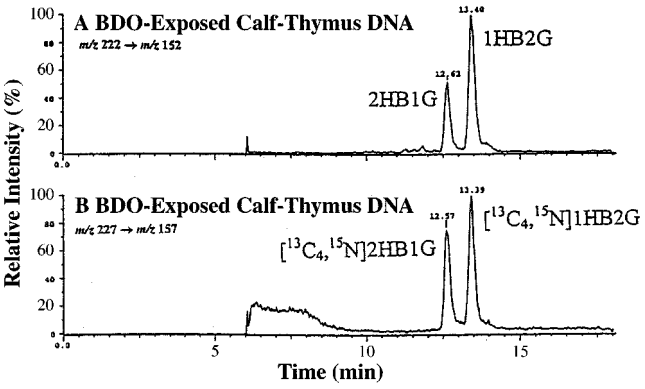

Figure 9. LC-SRM-MS chromatograms from analysis of 2HB1G and 1HB2G in a hydrolyzed BDO-exposed calf-thymus DNA sample to which the internal standard had been added. Panel A shows 2HB1G and 1HB2G, and panel B the isotope-labeled internal standards.

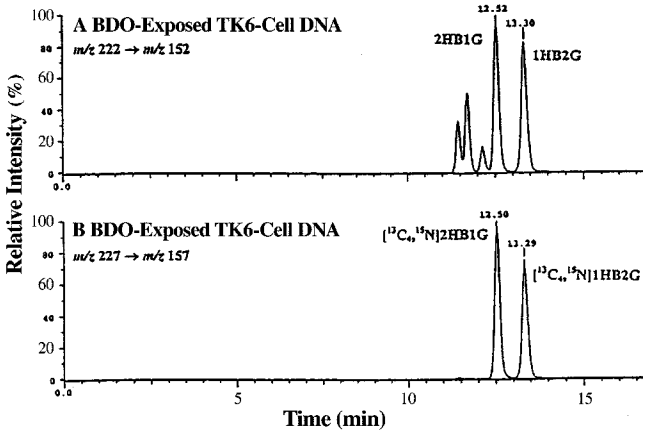

Figure 10. LC-SRM-MS chromatograms from analysis of 2HB1G and 1HB2G in a hydrolyzed BDO-exposed TK6-cell DNA sample to which the internal standard had been added. Panel A shows 2HB1G and 1HB2G, and panel B the isotope-labeled internal standards.

from the nominal concentration (accuracy) ranged from 93.1% to 112%. Interday precision for the 2HB1G QC samples ranged from 1.5% to 4.3%; deviation from the nominal concentration (accuracy) ranged from 96.7% to 105.2%. Interday precision for the 1HB2G QC samples ranged from 2.9% to 6.0%; deviation from the nominal concentration (accuracy) ranged from 93.5% to 98.9%.

LC-MS ASSAY FOR (±)-THBG IN CALF-THYMUS DNA

Calibration standards for (±)-THBG were analyzed in the range of 27.6 pg to 1386.0 pg. A typical calibration curve for (±)-THBG had a regression line of y = −0.0001 + 0.04x (r^2 = 0.999). A reagent blank, a control calf-thymus DNA sample, and a control calf-thymus DNA sample to which the internal standard was added confirmed that there were no interfering substances derived from the reagents or the DNA sample. A typical LC-SRM-MS chromatogram for the analysis of (±)-THBG in a BDO_2-exposed calf-thymus DNA sample is shown in Figure 11. The back-calculated calibration standards were within ±10% of the expected values.

VALIDATION OF LC-MS ASSAY FOR 2HB1G AND 1HB2G IN URINE

Calibration standards for 2HB1G and 1HB2G were analyzed in the range of 0.068 ng to 27.3 ng. A typical calibration curve for 2HB1G had a regression line of y = 0.0035 + 0.3240x (r^2 = 1.000); for 1HB2G, a typical regression line was y = 0.0027 + 0.2627x (r^2 = 1.000). The back-calculated calibration standards were within ±10% of the expected values. A reagent blank, a control urine sample for each species, and a control urine sample for each species to which only the internal standard had been added (Figure 12A) confirmed that no interfering substances were present in the reagents or the urine. A typical LC-SRM-MS chromatogram obtained for a BD-exposed mouse urine sample is shown in Figure 12B. Intraassay precision for the 2HB1G QC samples ranged from 0.01% to 7.29%; deviation from the nominal concentration (accuracy on each of 3 days) ranged from 84.48% to 97.48% (Table 1). Intraassay precision for the 1HB2G QC samples ranged from 0.01% to 9.61%; deviation from the nominal concentration (accuracy on each of 3 days) ranged from 83.33% to 118.72% (Table 2). Interday precision for the 2HB1G QC samples ranged from 4.83% to 7.28%; deviation from the nominal concentration (accuracy) ranged from 91.56% to 93.87% (Table 1). Interday precision for the 1HB2G QC samples ranged from 12.64% to 16.85%; deviation from the nominal concentration (accuracy) ranged from 103.92% to 105.77% (Table 2).

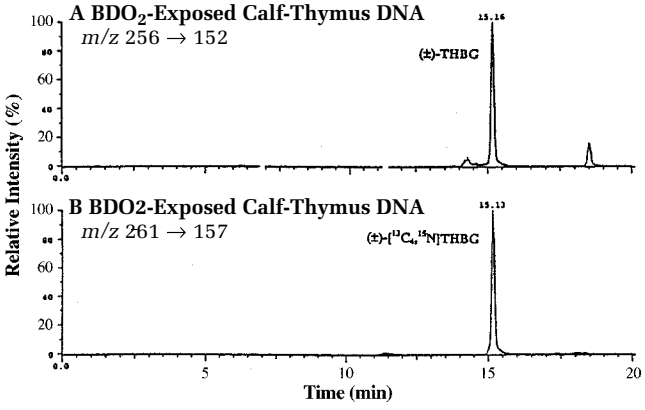

Figure 11. LC-SRM-MS chromatograms from analysis of (±)-THBG in a hydrolyzed BDO_2-exposed calf-thymus DNA sample to which the internal standard had been added. Panel A shows (±)-THBG, and panel B the isotope-labeled internal standard.

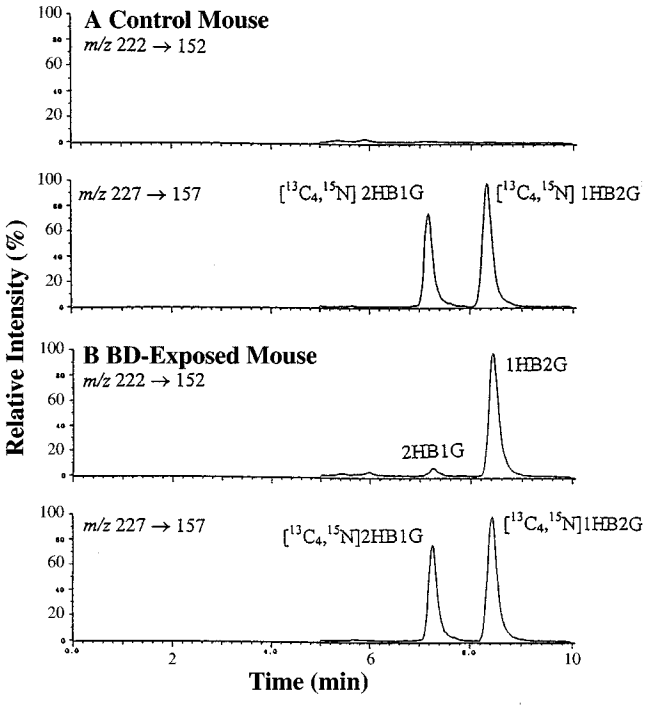

Figure 12. LC-SRM-MS chromatograms from analysis of 2HB1G and 1HB2G in urine from a control mouse (A) and a BD-exposed mouse (B). In each pair of chromatograms, the upper one shows 2HB1G and 1HB2G and the lower one shows the isotope-labeled internal standards.

VALIDATION OF LC-MS ASSAY FOR (±)-THBG IN URINE

Calibration standards for (±)-THBG were analyzed in the range of 0.276 ng to 37.6 ng. A typical calibration curve for (±)-THBG had a regression line of y = 0.0009 + 0.0578x (r^2 = 0.999). The back-calculated calibration standards were within ±10% of the expected values. A reagent blank, a control rat urine sample, a control mouse urine sample, a control rat urine sample to which only the internal standard had been added (Figure 13A), and a control mouse urine sample to which only the internal standard had been added (Figure 14A) confirmed that there were no interfering substances from the reagents or the urine at the retention time of (±)-THBG. Typical LC-SRM-MS chromatograms for a BD-exposed rat-liver DNA sample and a BD-exposed mouse-liver DNA sample are shown in Figures 13B and 14B, respectively. Intraassay precision ranged from 2.14% to 6.79%; deviation from the nominal concentration (accuracy) ranged from 98.20% to 110.70% (Table 3). Interday precision ranged from 2.59% to 6.30%; deviation from the nominal concentration (accuracy) ranged from 99.40% to 104.30% (Table 3).

Table 1. Precision and Accuracy for Analysis of 2HB1G in Urine from Mice and Rats[a]

	LLQ (0.136 ng/mL)	LQC (0.54 ng/mL)	MQC (4.36 ng/mL)	HQC (38.2 ng/mL)
Day 1[b]				
Mean	0.060	0.26	1.88	16.14
Precision (% RSD)	7.292	0.02	0.04	0.01
Accuracy (%)	88.88	95.64	86.03	84.48
n	6	5	5	5
Day 2				
Mean		0.25	2.10	18.62
Precision (% RSD)		0.05	0.03	0.02
Accuracy (%)		92.55	96.23	97.48
n		5	5	5
Day 3				
Mean		0.26	2.12	17.71
Precision (% RSD)		0.07	0.03	0.07
Accuracy (%)		93.43	97.43	92.73
n		5	5	5
Interday[c]				
Mean		0.26	2.03	17.49
Precision (% RSD)		4.83	6.29	7.28
Accuracy (%)		93.87	93.23	91.56
n		3	3	3

[a] Precision is the within-error expressed as the percentage relative to the SD (% RSD). Accuracy is the mean percentage of deviation from the expected value. Values are ng 2HB1G/mL urine.

[b] Replicate urine QC samples at the LLQ, LQC, MQC, and HQC were analyzed by LC-SRM-MS on a single day to determine the intraassay precision (% RSD) and accuracy (%).

[c] Replicate urine QC samples at the LQC, MQC, and HQC were analyzed on two additional days in order to obtain the interday precision (% RSD) and accuracy (%) across all three days.

Table 2. Precision and Accuracy for Analysis of 1HB2G in Urine from Mice and Rats[a]

	LLQ (0.136 ng/mL)	LQC (0.54 ng/mL)	MQC (4.36 ng/mL)	HQC (38.2 ng/mL)
Day 1[b]				
Mean	0.066	0.24	1.77	15.92
Precision (% RSD)	9.605	0.05	0.01	0.03
Accuracy (%)	97.366	101.93	98.48	83.33
n	6	5	5	5
Day 2				
Mean		0.30	2.56	22.68
Precision (% RSD)		0.05	0.01	0.01
Accuracy (%)		94.47	109.71	118.72
n		5	5	5
Day 3				
Mean		0.31	2.57	22.01
Precision (% RSD)		0.07	0.01	0.06
Accuracy (%)		94.22	107.32	115.25
n		5	5	5
Interday[c]				
Mean		0.28	2.30	20.20
Precision (% RSD)		12.64	16.85	16.05
Accuracy (%)		103.92	105.55	105.77
n		5	5	5

[a] Precision is the within-error expressed as the percentage relative to the SD (% RSD). Accuracy is the mean percentage of deviation from the expected value. Values are ng 1HB2G/mL urine.

[b] Replicate urine QC samples at the LLQ, LQC, MQC, and HQC were analyzed by LC-SRM-MS on a single day to determine the intraassay precision (% RSD) and accuracy (%).

[c] Replicate urine QC samples at the LQC, MQC, and HQC were analyzed on two additional days in order to obtain the interday precision (% RSD) and accuracy (%) across all three days.

VALIDATION OF LC-MS ASSAYS FOR 2HB1G, 1HB2G, AND (±)-THBG IN RAT- AND MOUSE-LIVER DNA

Calibration standards for 2HB1G and 1HB2G were analyzed in the range of 13.6 pg to 6,830.0 pg; for (±)-THBG, and *meso*-THBG, they were analyzed in the range of 69.0 pg to 13,800.0 pg. Typical calibration curves for 2HB1G ($y = 0.0080 + 0.00047x$; $r^2 = 0.996$), 1HB2G ($y = 0.0054 + 0.00059x$; $r^2 = 0.998$), (±)-THBG ($y = 0.0023 + 0.000059x$; $r^2 = 0.995$), and *meso*-THBG ($y = 0.0010 + 0.000067x$; $r^2 = 0.999$) are shown in Figure 15A and B and Figure 16A and B, respectively. The back-calculated calibration standards were within ±10% of the expected values. A reagent blank, a control rat-liver DNA sample, a control mouse-liver DNA sample, a control rat-liver DNA sample to which only [$^{13}C_4$,^{15}N]2HB1G and [$^{13}C_4$,^{15}N]1HB2G internal standards had been added (Figure 17A), a control rat-liver DNA sample to which only (±)-[$^{13}C_4$,^{15}N]THBG internal standard had been added (Figure 18A), a control mouse-liver DNA sample to which only [$^{13}C_4$,^{15}N]2HB1G and [$^{13}C_4$,^{15}N]1HB2G internal standards had been added (Figure 19A), and a control mouse-liver DNA sample to which only (±)-[$^{13}C_4$,^{15}N]THBG internal standard had been added (Figure 20A) confirmed that no interfering substances were present in the reagents or the tissue.

Typical LC-SRM-MS chromatograms for exposed rat-liver DNA samples are shown in Figure 17B (for 2HB1G and 1HB2G) and Figure 18B [for (±)-THBG and *meso*-THBG]. Typical LC-SRM-MS chromatograms for BD-exposed mouse-liver DNA samples are shown in Figure 19B (for 2HB1G and 1HB2G) and Figure 20B [for (±)-THBG and *meso*-THBG]. Intraassay precision for the 2HB1G QC samples ranged from 0.03% to 15.80%; deviation from the nominal concentration (accuracy) ranged from 89.67% to 106.23% (Table 4). Intraassay precision for the 1HB2G QC samples ranged from 0.02% to 5.52%; deviation from the nominal concentration (accuracy) ranged from 87.95% to

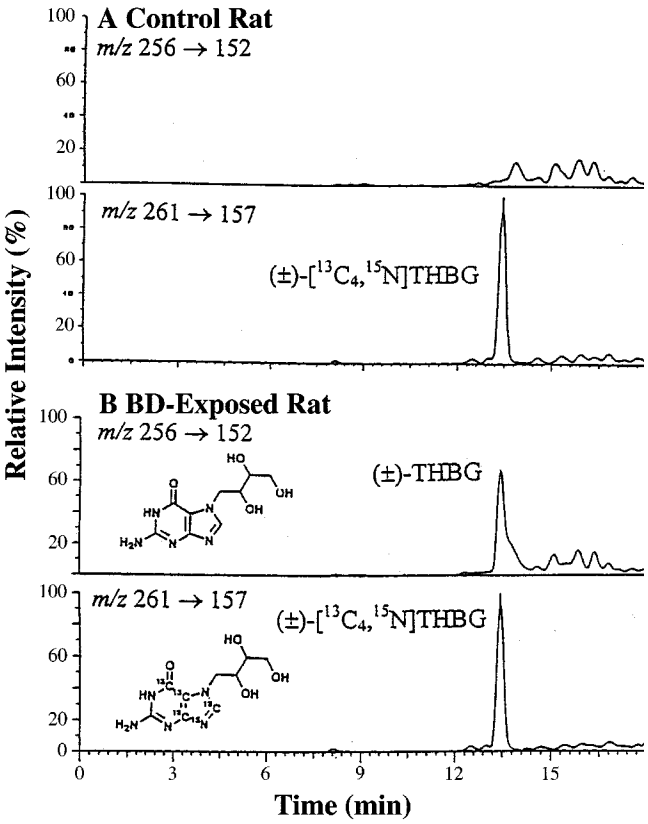

Figure 13. LC-SRM-MS chromatograms from analysis of (±)-THBG in urine from a control rat (A) and a BD-exposed rat (B). The internal standard had been added to both samples. In each pair of chromatograms, the upper one shows (±)-THBG and the lower one shows the isotope-labeled internal standard.

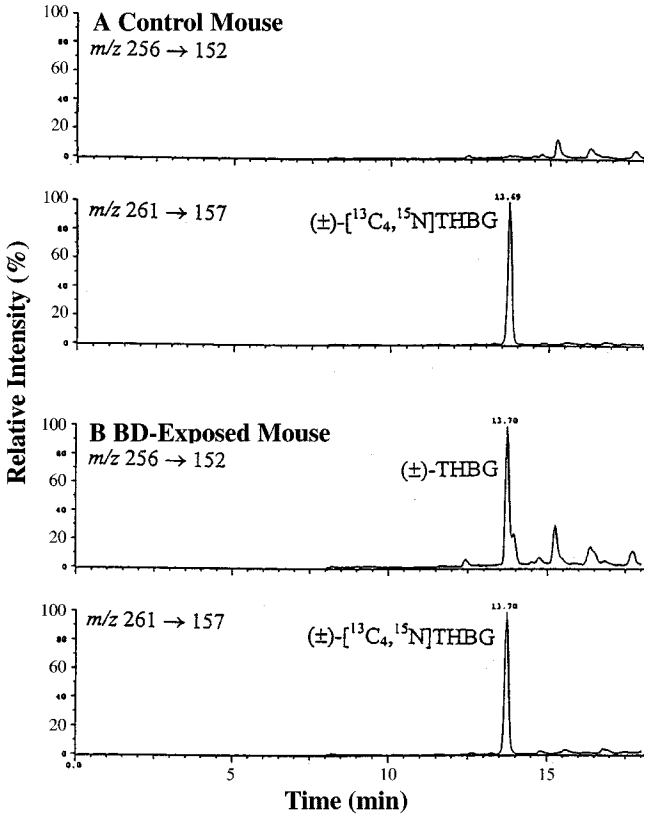

Figure 14. LC-SRM-MS chromatograms from analysis of (±)-THBG in urine from a control mouse (A) and a BD-exposed mouse (B). The internal standard had been added to both samples. In each pair of chromatograms, the upper one shows (±)-THBG and the lower one shows the isotope-labeled internal standard.

Table 3. Precision and Accuracy for Analysis of (±)-THBG in Urine from Mice and Rats[a]

	LLQ (0.138 ng/mL)	LQC (0.346 ng/mL)	MQC (1.38 ng/mL)	HQC (6.85 ng/mL)
Day 1[b]				
Mean	0.144	0.382	1.45	6.84
Precision (% RSD)	6.79	3.34	4.03	2.14
Accuracy (%)	104.3	110.7	105.1	99.0
n	6	6	6	6
Day 2				
Mean		0.348	1.36	6.79
Precision (% RSD)		4.41	4.86	3.09
Accuracy (%)		100.7	98.4	98.2
n		6	6	6
Day 3				
Mean		0.350	1.44	6.99
Precision (% RSD)		6.16	2.51	1.83
Accuracy (%)		101.4	104.2	101.1
n		6	6	6
Interday[c]				
Mean		0.360	1.42	6.87
Precision (% RSD)		6.30	4.72	2.59
Accuracy (%)		104.3	102.6	99.4
n		18	18	18

[a] Precision is the within-error expressed as the percentage relative to the SD (% RSD). Accuracy is the mean percentage of deviation from the expected value. Values are ng (±)-THBG/mL urine.

[b] Replicate urine QC samples at the LLQ, LQC, MQC, and HQC were analyzed by LC-SRM-MS on a single day to determine the intraassay precision (% RSD) and accuracy (%).

[c] Replicate urine QC samples at the LQC, MQC, and HQC were analyzed on two additional days in order to obtain the interday precision (% RSD) and accuracy (%) across all three days.

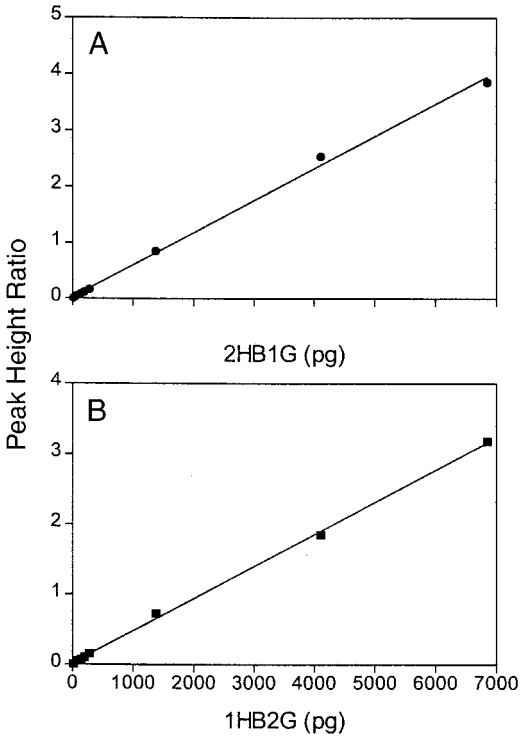

Figure 15. Typical calibration curves for hydrolyzed rat- and mouse-liver DNA in the range of 13.6 pg to 6,830.0 pg. The curve for 2HB1G (A) shows regression line y = 0.0080 + 0.00047x, r^2 = 0.996; the curve for 1HB2G (B) shows regression line y = 0.0054 + 0.00059x; r^2 = 0.998.

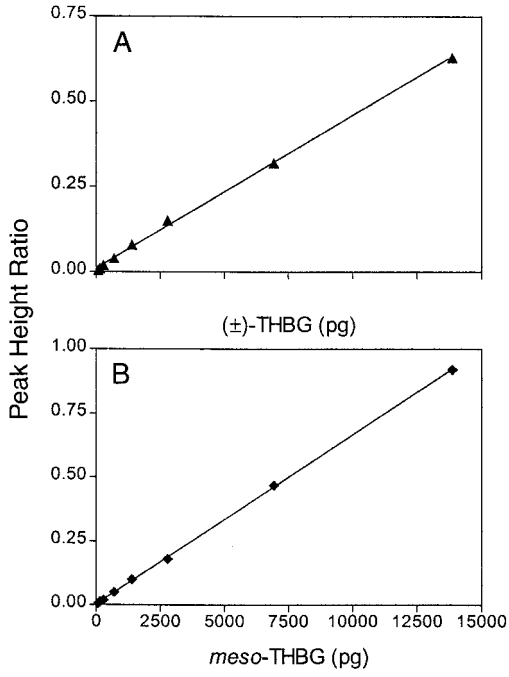

Figure 16. Typical calibration curves for hydrolyzed rat- and mouse-liver DNA in the range of 69.0 pg to 13,800.0 pg. The curve for (±)-THBG (A) shows regression line y = 0.00227 + 0.000059x, r^2 = 0.995; the curve for *meso*-THBG (B) shows regression line y = 0.0010 + 0.000067x; r^2 = 0.999.

112.63% (Table 5). Intraassay precision for the (±)-THBG QC samples ranged from 2.1% to 14.7%; deviation from the nominal concentration (accuracy) ranged from 95.90% to 110.1% (Table 6). Interday precision for the 2HB1G QC samples ranged from 5.48% to 8.37%; deviation from the nominal concentration (accuracy) ranged from 91.86% to 101.41% (Table 4). Interday precision for the 1HB2G QC samples ranged from 6.59% to 9.14%; deviation from the nominal concentration (accuracy) ranged from 96.87% to 108.96% (Table 5). Interday precision for the (±)-THBG QC samples ranged from 3.2% to 7.8%; deviation from the nominal concentration (accuracy) ranged from 102.7% to 107.3% (Table 6).

FORMATION OF BDO-GUANINE ADDUCTS IN CALF-THYMUS DNA

Exposure of calf-thymus DNA to BDO for 24 hours resulted in the formation of equimolar amounts of 1HB2G and 2HB1G (see Figure 9). In the presence of a 40-fold molar excess of BDO relative to the amount of nucleotide, DNA-adduct concentrations of 1HB2G and 2HB1G reached 448 adducts/1 × 10^6 normal bases (n = 2) and 438 adducts/1 × 10^6 normal bases (n = 2), respectively. Spontaneous depurination resulted in the appearance of these DNA adducts in the incubation medium. The concentrations of 2HB1G and 1HB2G in the medium increased almost linearly over the 48 hours of incubation. After 1 hour of incubation, 3.30 pmol of 2HB1G (n = 2) and 4.0 pmol of 1HB2G (n = 2) were found in the supernatant. This increased to 54.50 pmol of 2HB1G (n = 2) and 51.90 of 1HB2G (n = 2) after 12 hours of incubation. The amounts of 2HB1G started to plateau after 48 hours, when 233.1 pmol of 2HB1G (n = 2) and 226.1 pmol of 1HB2G (n = 2) were present in the incubation medium. Almost all of the BDO-derived adducts were lost from the DNA after 96 hours of incubation, at which point 245.5 pmol of 2HB1G (n = 2) and 237.1 pmol of 1HB2G (n = 2) were present in the incubation medium.

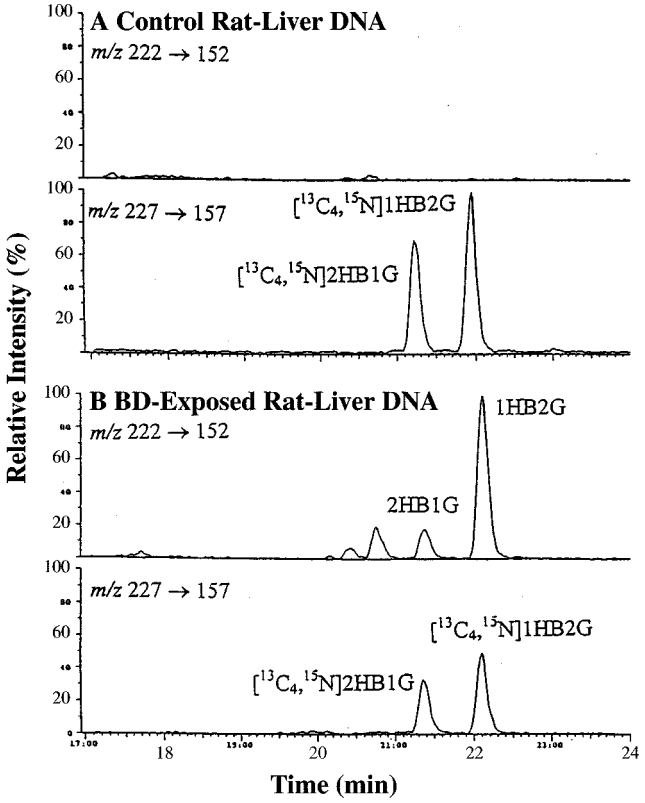

Figure 17. LC-SRM-MS chromatograms from analysis of 2HB1G and 1HB2G in hydrolyzed rat-liver DNA samples. A control sample (A) and a sample from a rat exposed to BD for 10 days (B); the internal standards had been added to both samples. In each pair of chromatograms, the upper one shows 2HB1G and 1HB2G and the lower one shows the isotope-labeled internal standards.

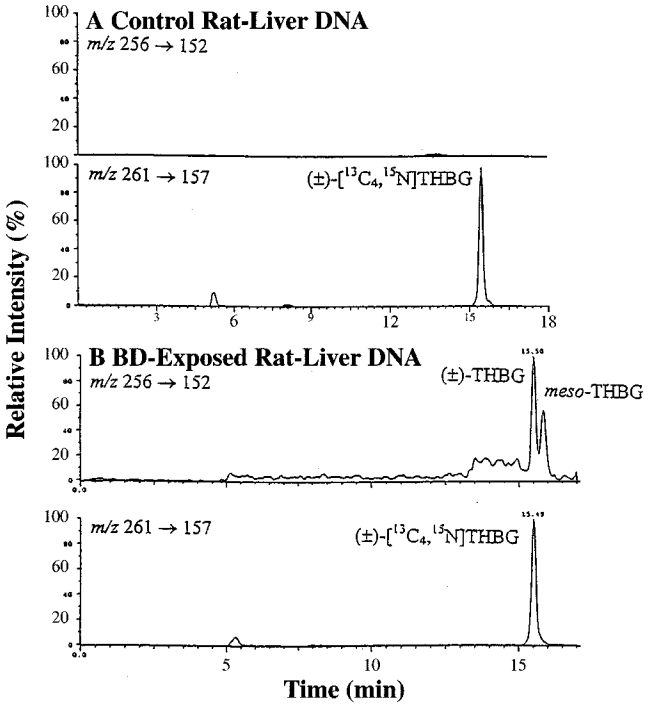

Figure 18. LC-SRM-MS chromatograms from analysis of THBG in hydrolyzed rat-liver DNA samples. A control sample (A) and a sample from a rat exposed to BD for 10 days (B); the internal standard had been added to both samples. In each pair of chromatograms, the upper one shows (±)-THBG and *meso*-THBG and the lower one shows the isotope-labeled internal standard.

FORMATION OF BDO-GUANINE ADDUCTS IN TK6-CELL DNA

Exposure of TK6 cells to 400 µM BDO for 24 hours also resulted in the formation of equimolar amounts of 1HB2G and 2HB1G (see Figure 10). At the end of the exposure period, the concentration of 1HB2G was 4.3 ± 0.9 adducts/1×10^6 normal cells ($n = 4$); the concentration of 2HB1G was 4.1 ± 1.0 adducts/1×10^6 normal cells ($n = 2$). After incubation for a further 72 hours, the concentrations of 1HB2G and 2HB1G had dropped to 0.70 ± 0.4 adducts/1×10^6 normal cells ($n = 2$) and 1.10 ± 0.70 adducts/1×10^6 normal cells ($n = 2$), respectively. Both 2HB1G (6.82 ± 1.44 pmol; $n = 2$) and 1HB2G (6.48 ± 1.56 pmol; $n = 2$) were detected in the cell supernatant after 24 hours. After removal of BDO and incubation for an additional 72 hours, the amounts in supernatant increased to 13.69 ± 0.41 pmol ($n = 2$) and 9.72 ± 0.41 pmol ($n = 4$) 2HB1G and 1HB2G, respectively.

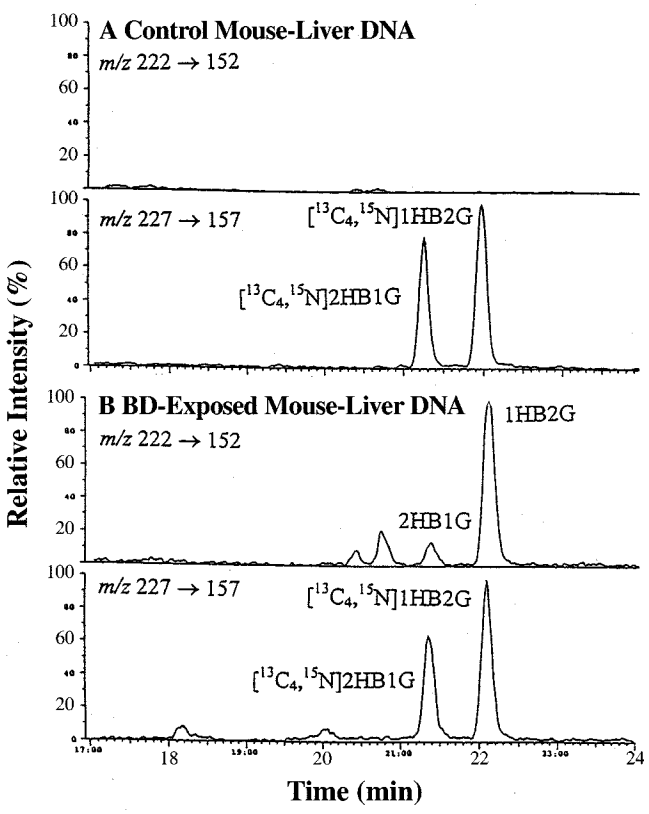

Figure 19. LC-SRM-MS chromatograms from analysis of 2HB1G and 1HB2G in hydrolyzed mouse-liver DNA samples. A control sample (A) and a sample from a mouse exposed to BD for 10 days (B); the internal standards had been added to both samples. In each pair of chromatograms, the upper one shows 2HB1G and 1HB2G and the lower one shows the isotope-labeled internal standards.

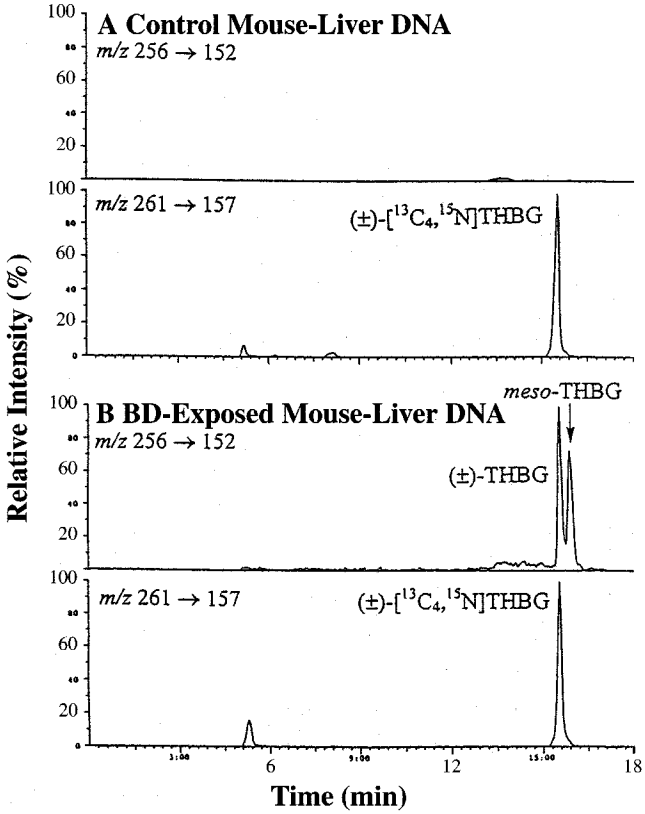

Figure 20. LC-SRM-MS chromatograms from analysis of THBG in hydrolyzed mouse-liver DNA samples. A control sample (A) and a sample from a mouse exposed to BD for 10 days (B); the internal standard had been added to both samples. In each pair of chromatograms, the upper one shows (±)-THBG and *meso*-THBG and the lower one shows the isotope-labeled internal standard.

FORMATION OF BDO_2-GUANINE ADDUCTS IN CALF-THYMUS DNA

A 4-hour exposure of calf-thymus DNA (100 µg, 300 nmol DNA bases) to excess BDO_2 (872 nmol) resulted in the formation of (±)-THBG (22.4 pmol, 67 adducts/1×10^6 DNA normal bases) (Figure 21). The (±)-THBG was detected after HCl acid hydrolysis of the DNA and after acid hydrolysis of the aqueous NH_4OAc/ethanol supernatant after DNA precipitation (see Figure 21). Acid hydrolysis with HCl resulted in the formation of the chlorohydrin and (bis)guanine adducts. However, hydrolysis with formic acid resulted in elimination of the chlorohydrin together with a substantial decrease in the amount of (bis)guanine adduct compared with the amount of THBG (data not shown).

Table 4. Precision and Accuracy for Analysis of 2HB1G in Rat- and Mouse-Liver DNA[a]

	LLQ (13.6 pg)	LQC (27 pg)	MQC (109 pg)	HQC (190 pg)
Day 1[b]				
Mean	12.4	26.1	102.18	202.90
Precision (% RSD)	15.8	0.08	0.03	0.06
Accuracy (%)	91.36	95.72	93.74	106.23
n	5	5	5	5
Day 2				
Mean		24.48	106.90	187.15
Precision (% RSD)		0.03	0.05	0.08
Accuracy (%)		89.67	98.07	97.98
n		5	5	5
Day 3				
Mean		24.51	106.83	191.03
Precision (% RSD)		0.06	0.07	0.10
Accuracy (%)		89.77	98.01	100.02
n		5	5	5
Interday[c]				
Mean		25.1	105.30	193.70
Precision (% RSD)		6.75	5.48	8.37
Accuracy (%)		91.86	96.61	101.41
n		3	3	3

[a] Precision is the within-error expressed as the percentage relative to the SD (% RSD). Accuracy is the mean percentage of deviation from the expected value.

[b] Replicate QC samples at the LLQ, LQC, MQC, and HQC were analyzed by LC-SRM-MS on a single day to determine the intraassay precision (% RSD) and accuracy (%).

[c] Replicate QC samples at the LQC, MQC, and HQC were analyzed on two additional days in order to obtain the interday precision (% RSD) and accuracy (%) across all three days.

Table 5. Precision and Accuracy for Analysis of 1HB2G in Rat- and Mouse-Liver DNA[a]

	LLQ (13.6 pg)	LQC (27 pg)	MQC (109 pg)	HQC (190 pg)
Day 1[b]				
Mean	11.96	27.83	107.34	200.48
Precision (% RSD)	5.52	0.09	0.13	0.10
Accuracy (%)	87.95	101.93	98.48	104.96
n	5	5	5	5
Day 2				
Mean		25.79	119.58	215.13
Precision (% RSD)		0.08	0.05	0.02
Accuracy (%)		94.47	109.71	112.63
n		5	5	5
Day 3				
Mean		25.72	116.98	208.73
Precision (% RSD)		0.10	0.07	0.05
Accuracy (%)		94.22	107.32	109.28
n		5	5	5
Interday[c]				
Mean		26.45	114.63	208.11
Precision (% RSD)		8.94	9.14	6.59
Accuracy (%)		96.87	105.17	108.96
n		5	5	5

[a] Precision is the within-error expressed as the percentage relative to the SD (% RSD). Accuracy is the mean percentage of deviation from the expected value.

[b] Replicate QC samples at the LLQ, LQC, MQC, and HQC were analyzed by LC-SRM-MS on a single day to determine the intraassay precision (% RSD) and accuracy (%).

[c] Replicate QC samples at the LQC, MQC, and HQC were analyzed on two additional days in order to obtain the interday precision (% RSD) and accuracy (%) across all three days.

BDO-GUANINE AND BDO_2-GUANINE ADDUCTS IN URINE OF MICE AND RATS EXPOSED TO BD

The amount of 1HB2G in 24-hour rat urine samples after exposure to BD for 10 days increased from 161.9 ng on day 1 after exposure to 229.2 ng on day 2. 1HB2G then decreased to 201.4 ng on day 3 (total of 592.5 ng over 72 hours) (see Figure 22A). The amount of 1HB2G in 24-hour mouse urine samples increased from 83.8 ng on day 1 after exposure to 110.8 ng on day 2; it then decreased to 86.6 ng on day 3 (total of 281.2 ng over 72 hours) (see Figure 22B).

Table 6. Precision and Accuracy for Analysis of (±)-THBG in Rat- and Mouse-Liver DNA[a]

	LLQ (69.3 pg)	LQC (138.6 pg)	MQC (1,386.0 pg)	HQC (1,3860.0 pg)
Day 1[b]				
Mean	73.2	144.8	1336.5	14544.6
Precision (% RSD)	14.7	5.8	3.6	2.1
Accuracy (%)	105.6	104.5	96.4	104.9
n		6	6	6
Day 2				
Mean		133.0	1526.4	15034.6
Precision (% RSD)		8.6	5.9	2.9
Accuracy (%)		95.9	110.1	108.5
n		6	6	6
Day 3				
Mean		149.1	1512.0	15026.9
Precision (% RSD)		4.8	2.1	3.5
Accuracy (%)		107.6	109.1	108.4
n		6	6	6
Interday[c]				
Mean		142.3	1458.3	14868.7
Precision (% RSD)		7.8	7.3	3.2
Accuracy (%)		102.7	105.2	107.3
n		3	3	3

[a] Precision is the within-error expressed as the percentage relative to the SD (% RSD). Accuracy is the mean percentage of deviation from the expected value.

[b] Replicate QC samples at the LLQ, LQC, MQC, and HQC were analyzed by LC-SRM-MS on a single day to determine the intraassay precision (% RSD) and accuracy (%).

[c] Replicate QC samples at the LQC, MQC, and HQC were analyzed on two additional days in order to obtain the interday precision (% RSD) and accuracy (%) across all three days.

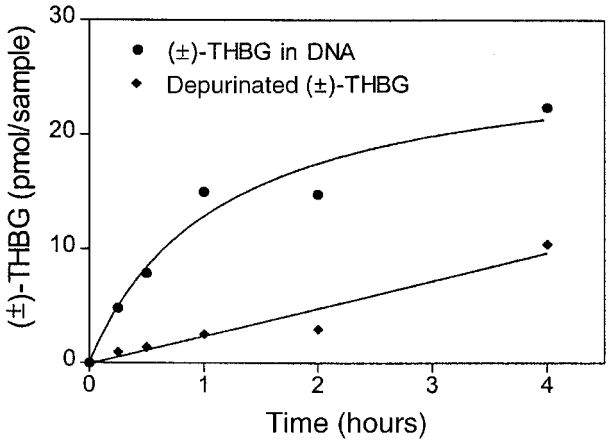

Figure 21. Formation of (±)-THBG in calf-thymus DNA exposed for 4 hours to BDO_2 and appearance of (±)-THBG in supernatant. The filled circles (●) represent (±)-THBG that was present in the DNA, and the filled diamonds (◆) represent depurinated (±)-THBG that was present in the supernatant.

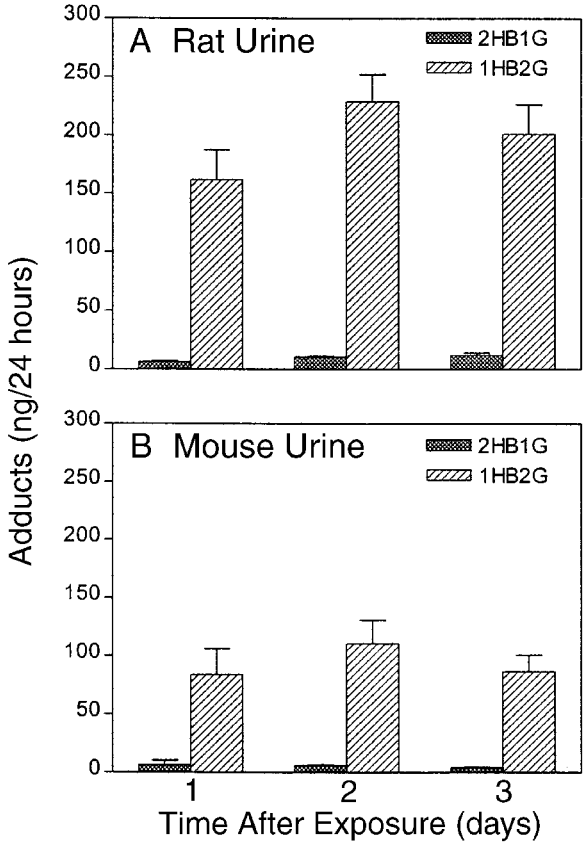

Figure 22. Amounts of 2HB1G and 1HB2G adducts in urine on each of three days after exposure to BD. The graphs represent adduct amounts in 24-hour urine samples from rats (A) and mice (B) exposed to BD for 10 days. 2HB1G is shown in cross-hatched columns, and 1HB2G is shown in diagonally striped columns. Results are means ± SD ($n = 4$) for each time point. Urinary excretion of 2HB1G and 1HB2G by rats was significantly different ($p < 0.05$; Student t test) compared with excretion by mice on each of the 3 days.

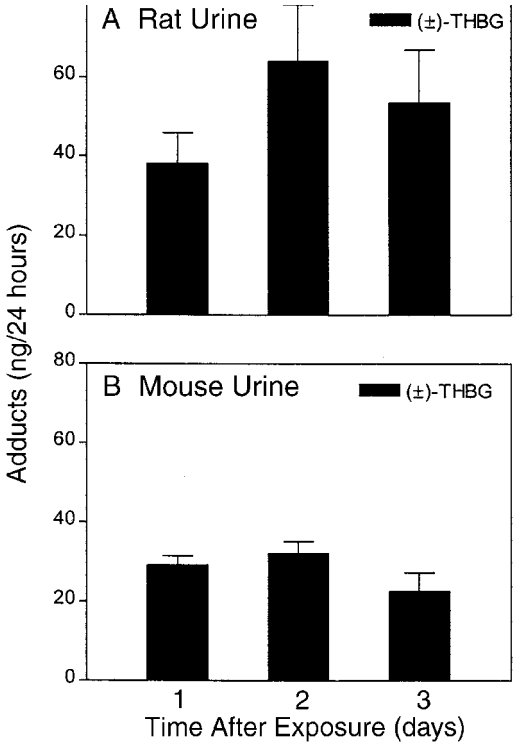

Figure 23. Amounts of (±)-THBG adducts in urine on each of three days after exposure to BD. The graphs represent adduct amounts in 24-hour urine samples from rats (A) and mice (B) exposed to BD for 10 days. Results are means ± SD ($n = 4$) for each time point. Urinary excretion of (±)-THBG by rats was significantly different ($p < 0.05$; Student t test) from excretion by mice on each of the 3 days.

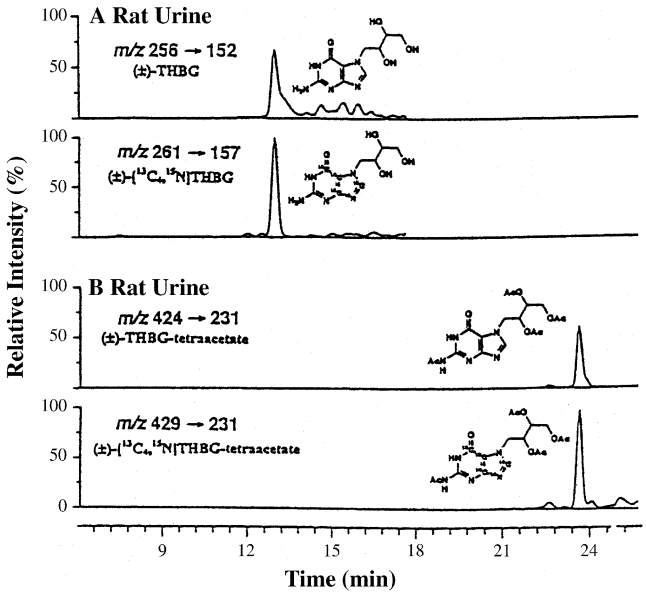

Figure 24. LC-SRM-MS chromatograms from analysis of (±)-THBG isolated from the urine of BD-exposed rats. (±)-THBG (A) and the tetraacetate derivative of (±)-THBG (B). In each pair of chromatograms, the upper one shows the adduct and the lower one shows the isotope-labeled internal standard.

The amount of 2HB1G in 24-hour rat urine samples increased from 6.6 ng on day 1 after exposure to 10.7 ng on day 2. It then increased slightly to 12.3 ng on day 3 (total of 29.5 ng over 72 hours) (Figure 22A). The mouse urine 2HB1G decreased slightly from 6.6 ng on day 1 to 6.0 ng on day 2; it then decreased slightly to 4.3 ng on day 3 (total of 16.9 ng over 72 hours) (Figure 22B).

The amount of (±)-THBG in 24-hour rat urine samples increased from 38.3 ng on day 1 to 64.1 ng on day 2. It then decreased to 53.7 ng on day 3 (total of 156.1 ng over 72 hours) (Figure 23A). The amount of (±)-THBG in 24-hour mouse urine samples increased from 29.3 ng on day 1 to 32.2 ng on day 2; it then decreased to 22.7 ng on day 3 (total of 84.2 ng over 72 hours) (Figure 23B). The THBG isolated from rat urine was analyzed by LC-SRM-MS (Figure 24A), acetylated, and reanalyzed by LC-SRM-MS as the tetraacetate derivative (Figure 24B). No additional peaks were observed in the chromatogram (see Figure 24B). A urine sample from a BD-exposed mouse was reanalyzed using a slower gradient system than had been employed in the quantitation. The control urine again showed no interfering substances at the retention time of (±)-THBG in the LC-SRM-MS chromatogram. Urine from a BD-exposed mouse showed a signal from (±)-THBG at 15.60 minutes, together with an additional signal at 16.05 minutes, in the LC-SRM-MS chromatogram (Figure 25A). This signal increased in intensity when authentic *meso*-THBG was added to the sample (Figure 25B).

BDO-GUANINE AND BDO_2-GUANINE ADDUCTS IN LIVER DNA OF MICE AND RATS EXPOSED TO BD

The concentration of 1HB2G in rat-liver DNA increased from 0.7 adducts/1 × 10^6 normal bases on day 1 of exposure to 2.3 adducts/1 × 10^6 normal bases on day 10 of exposure; it then declined to 0.4 adducts/1 × 10^6 normal bases on day 6 after exposure (Figure 26A). Concentrations of 1HB2G in mouse-liver DNA increased from 0.6 adducts/1 × 10^6 normal bases on day 1 of exposure to 4.6 adducts/1 × 10^6 normal bases on day 10 of exposure. It then declined to 0.9 adducts/1 × 10^6 normal bases on day 6 after exposure (Figure 26B). The concentration of 2HB1G in rat-liver DNA increased from 0.05 adducts/1 × 10^6 normal bases on day 1 of exposure to 0.31 adducts/1 × 10^6 normal bases on day 10 of exposure. It then also declined to 0.08 adducts/1 × 10^6 normal bases on day 6 after exposure (see Figure 26A). The concentration of 2HB1G in mouse-liver DNA increased from 0.11 adducts/1 × 10^6 normal bases on day 1 of exposure to 1.00 adducts/1 × 10^6 normal bases on day 10 of exposure. It then declined to 0.34 adducts/1 × 10^6 normal bases on day 6 after exposure (see Figure 26B).

The concentration of (±)-THBG in rat-liver DNA increased from 0.13 adducts/1 × 10^6 normal bases on day 1 of exposure to 1.64 adducts/1 × 10^6 normal bases on day 10 of exposure. The concentration of (±)-THBG declined to 0.27 adducts/1 × 10^6 normal bases on day 6 after exposure (Figure 27A). The concentration of (±)-THBG in mouse-liver DNA increased from 1.3 adducts/1 × 10^6 normal bases on day 1 of exposure to 3.9 adducts/1 × 10^6 normal bases on day 10 of exposure; it declined to 1.2 adducts/ 1 × 10^6 normal bases on day 6 after exposure (Figure 27B). THBG isolated from the BD-exposed rat-liver DNA was acetylated and reanalyzed by LC-SRM-MS as the tetraacetate derivative. An additional peak that was derived from the tetraacetate derivative of *meso*-THBG was observed in the chromatogram (Figure 28B). This confirmed the presence of *meso*-THBG in the liver DNA samples. The concentration of *meso*-THBG in rat-liver DNA increased from 0.10 adducts/1 × 10^6 normal bases on day 1 of exposure to 0.78 adducts/1 × 10^6 normal bases on day 10 of exposure. The concentration then declined to 0.20 adducts/1 × 10^6 normal bases on day 6 after exposure (see Figure 27A). The concentration of *meso*-THBG in mouse-liver DNA increased from 0.6 adducts/1 × 10^6 normal bases on day 1 of exposure to 2.2 adducts/1 × 10^6 normal bases on day 10 of exposure. This concentration then declined to 1.0 adducts/1 × 10^6 normal bases on day 6 after exposure (see Figure 27B).

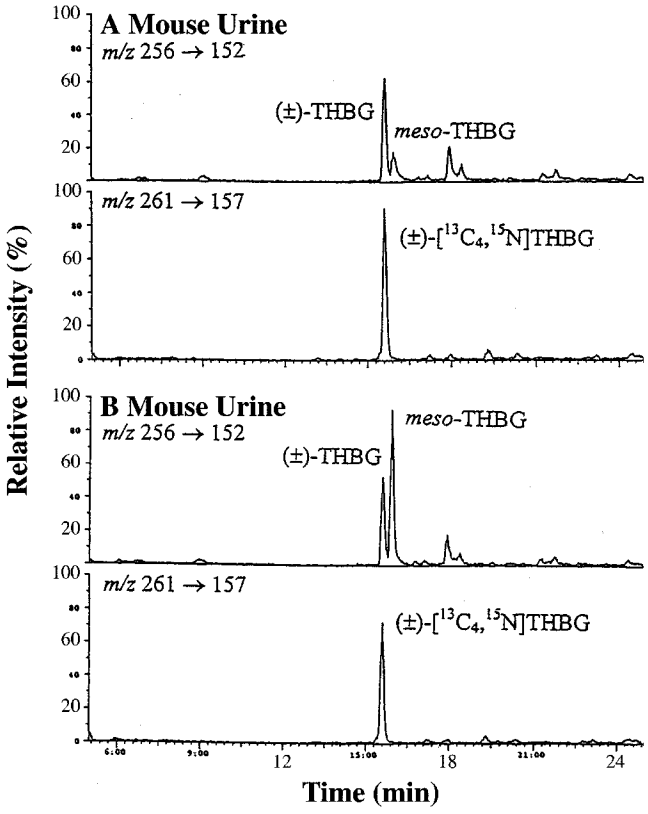

Figure 25. LC-SRM-MS chromatograms from analysis of THBG in urine samples from a BD-exposed mouse. One sample (A) to which only the internal standard had been added; and another sample (B) to which the internal standard had been added after authentic *meso*-THBG had been added. In each pair of chromatograms, the upper one shows (±)-THBG and *meso*-THBG and the lower one shows the isotope-labeled internal standard.

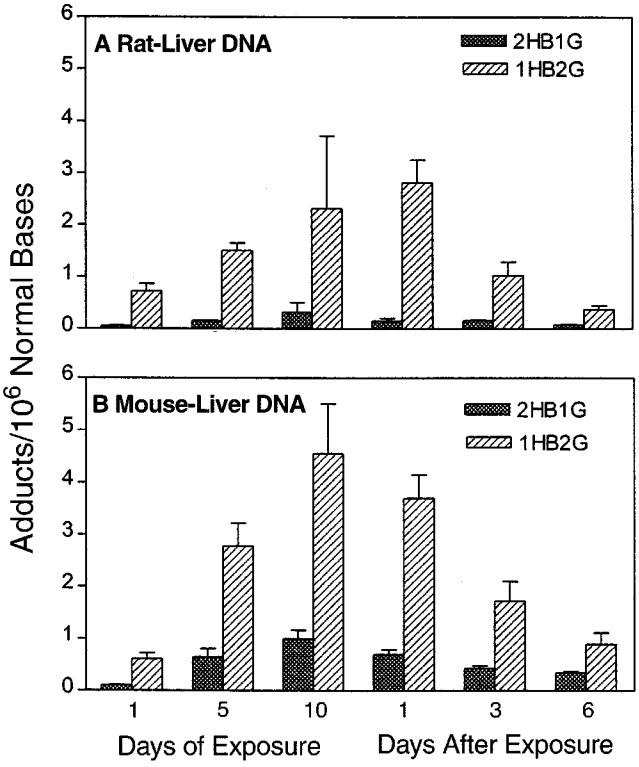

Figure 26. Amounts of 2HB1G and 1HB2G in hydrolyzed liver DNA. The graphs represent adduct amounts in liver from rats (A) and mice (B) exposed to BD for 10 days and monitored for 6 days after exposure. 2HB1G is shown in cross-hatched columns; 1HB2G is shown in diagonally striped columns. Results are means ± SD ($n = 5$) for each time point. The amounts of 2HB1G and 1HB2G in hydrolyzed rat-liver DNA were significantly different ($p < 0.05$; Student t test) from the amounts in hydrolyzed mouse-liver DNA at each time point except for day 1 after exposure.

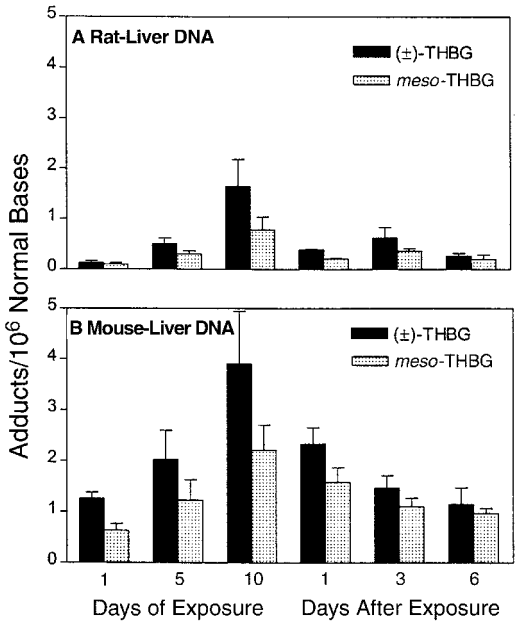

Figure 27. Amounts of (±)-THBG and *meso*-THBG in hydrolyzed liver DNA. The graphs represent amounts in liver from rats (A) and mice (B) exposed to BD for 10 days and monitored for 6 days after exposure. (±)-THBG is shown in dark columns and *meso*-THBG is shown in dotted columns. Results are means ± SD (n = 5) for each time point. At each time point, the concentrations of (±)-THBG and *meso*-THBG in hydrolyzed rat-liver DNA were significantly different ($p < 0.05$; Student t test) from the amounts in hydrolyzed mouse-liver DNA.

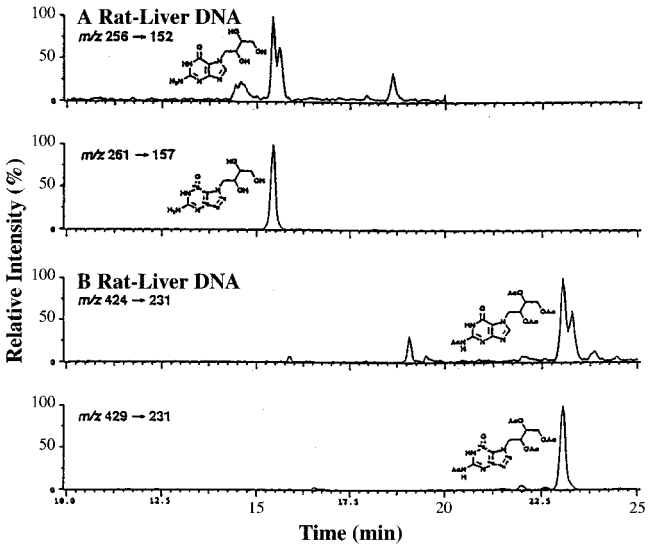

Figure 28. LC-SRM-MS chromatograms from analysis of THBG isolated from hydrolyzed liver DNA from a BD-exposed rat. (±)-THBG (A) and the tetraacetate derivatives of (±)-THBG and *meso*-THBG (B). In each pair of chromatograms, the upper one shows the adduct or adducts and the lower one shows the isotope-labeled internal standard. Panel A top: (±)-THBG; panel A bottom: (±)-[$^{13}C_4{}^{15}N$]THBG. Panel B top: (±)-THBG-tetraacetate and *meso*-THBG-tetraacetate; panel B bottom: (±)-[$^{13}C_4{}^{15}N$]THBG-tetraacetate.

DISCUSSION

Compelling evidence indicates that BD requires metabolic activation before it is able to covalently modify DNA. Three BD metabolites (BDO, BDO_2, and BDO-diol) have been identified as potential ultimate carcinogens that could react directly with DNA (see Figure 1). The present study has focused on the characterization and quantitation of N7-adducts of guanine derived from these metabolites because they could provide insight into the potentially genotoxic dose to which the DNA had been subjected. N7-Adducts of guanine can be repaired from the DNA strand through the action of glycosylases or through excision repair. Alternatively, adducts can be released from DNA through spontaneous depurination to produce abasic sites. These processes can then result in the loss of covalently modified bases from the DNA and to their appearance in an accessible biological fluid such as urine (Shukur and Farmer 1992). Urinary adducts reflect both spontaneous depurination and repair as has been postulated for the N7-guanine adducts derived from other genotoxins such as aflatoxin B_1 (AFB_1) (Groopman et al. 1992), benzo[*a*]pyrene (Authrup and Seremet 1986), dimethylnitrosamine (Craddock and Magee 1967), and ethylene dibromide (Kim and Guengerich 1989). Investigators reasoned that concentrations of the urinary N7-guanine adducts may serve as an index of an integrated whole-body exposure to BD (Shukur and Farmer 1992). LC-MS is rapidly becoming accepted as the method of choice for the quantitative and structural analysis of DNA adducts (Chaudhary et al. 1995). Intense signals corresponding to MH^+ are observed, and structurally informative product ions are observed when CID is performed. The present study has confirmed the utility of ESI and LC-MS for the specific and sensitive analysis of BD-derived DNA adducts.

A 24-hour treatment of calf-thymus DNA with BDO (the initially formed oxidative metabolite of BD) resulted in a time-dependent formation of two DNA adducts, 2HB1G and 1HB2G. BDO contains one chiral center such that the 2HB1G and 1HB2G regioisomers that were formed each consisted of an enantiomeric pair. Although the regioisomers could be readily separated by LC-MS (see Figure 9), the individual enantiomeric pairs eluted together. Approximately equal amounts of 2HB1G and 1HB2G were observed after a 24-hour exposure as determined by LC-MS. This indicated that regioselectivity did not occur in the reaction of calf-thymus DNA with BDO. Spontaneous depurination of the DNA occurred when BDO-treated calf-thymus DNA was incubated at 37°C for 24 hours in water. Increasing amounts of 2HB1G and 1HB2G were observed

in the incubation medium when the incubation was extended for a further 72 hours. No regioselectivity in the DNA bases formed by spontaneous depurination was noted; equal amounts of 2HB1G and 1HB2G were observed at all time points. A similar experiment was performed in an in vitro cell system using TK6 lymphoblastoid cells, which were exposed to 400 mM BDO. Again, approximately equal amounts of 2HB1G and 1HB2G were observed in the DNA (see Figure 10), suggesting that no regioselectivity occurred in the reaction of TK6-cell DNA with BDO. Spontaneous depurination of the DNA occurred during the whole time course of the incubation; at 24 hours, slightly greater amounts of 2HB1G and 1HB2G were observed in the cell supernatant than in the DNA.

Analysis of the N7-guanine adduct derived from BDO_2 was complicated by the need to perform a hydrolysis of the initially formed epoxy hydroxy adduct to produce the (±)THBG adduct that was expected to form in vivo (see Figure 3). An incubation of calf-thymus DNA with (±)-BDO_2 resulted in the formation of (±)-THBG in the hydrolyzed DNA. After 4 hours, it was possible to show that depurination occurred because (±)-THBG appeared in the hydrolyzed supernatant (see Figure 21). In a separate experiment, the DNA hydrolysate was analyzed for (±)-THBG, the corresponding hydroxy epoxide, chlorohydrin, acetate, and (bis)guanine adduct. Substantial quantities of each of these compounds were observed when the hydrolysis was performed with HCl (data not shown). However, (±)-THBG was the major product when formic acid was employed for the DNA hydrolysis; under these conditions, only a trace amount of the (bis)guanine adduct appeared. The (bis)guanine adduct most likely arose from a reaction between the hydroxy epoxide and the guanine released by acid hydrolysis of the DNA. In light of these observations, formic acid was used for the hydrolysis of liver-DNA samples from animals exposed to BD.

The data from in vitro incubations of DNA with BDO or BDO_2 indicated that spontaneous depurination represented an important pathway by which 2HB1G, 1HB2G, and THBG could be removed from DNA. This, taken together with the possibility that repair of the covalently modified DNA bases by glycosylases and excision-repair enzymes could occur, indicated a high probability that the DNA adducts would be excreted in urine. Previous studies have analyzed urinary alkylated purines as an index of exposure of DNA to alkylating agents (Shukur and Farmer 1992). Perhaps the most convincing data exist for AFB_1, where a clear link has been established in animal models between AFB_1-guanine adduct concentrations in urine and in liver (Benner et al. 1981). This has allowed urinary AFB_1-guanine to be used extensively as a biomarker of risk for AFB_1-mediated carcinogenesis in human subjects (Groopman et al. 1992). Therefore, urinary DNA adducts can provide useful information on exposure to alkylating carcinogens.

Urine is the biological fluid of choice as it can be readily collected from normal subjects, it is easy and safe to handle, and it can be readily transported to the site of analysis without the need for excessive precautions (Shukur and Farmer 1992). The validated assay for urinary 2HB1G and 1HB2G was applied to the analysis of DNA adducts in the urine of rats and mice exposed to 1,250 ppm BD for 10 days. Urinary excretion of 1HB2G on each of the three days after BD exposure increased significantly more ($p < 0.05$) in the rat than in the mouse (see Figure 22). Surprisingly, much less of the other regioisomer (2HB1G) was excreted in both animal species. However, urinary excretion of 2HB1G on day 2 and day 3 after BD exposure increased significantly ($p < 0.05$) in the rat compared with excretion in the mouse (see Figure 22).

The assay for urinary (±)-THBG employed PBA columns, which served to promote formation of complexes involving the diols present in the N7-trihydroxybutyl group of the THBG molecule. Because this polar compound bound poorly to reverse-phase SPE columns, removing interfering substances from urine was difficult with conventional clean-up methods. However, the specificity of the PBA columns for diols resulted in an efficient clean-up of urinary THBG. Almost twice as much (±)-THBG was found in the urine of rats as in the urine of mice. This finding was similar to what had been observed with urinary DNA adducts derived from BDO. Excretion of (±)-THBG was greatest on day 2 after exposure and started to decline on day 3 for both rats and mice. The increased urinary excretion of (±)-THBG by the rat reached statistical significance ($p < 0.05$) on each of the three days monitored after BD exposure (see Figure 23).

The LC-SRM-MS chromatogram obtained from urine of rats exposed to BD (see Figure 24A) showed a slight shoulder on the side of the (±)-THBG peak. A signal was observed, however, at the same retention time as the shoulder in the control rat-urine sample (see Figure 13A). Therefore, this shoulder peak was thought to derive from an interfering endogenous component of rat urine. To confirm this observation, a tetraacetate derivative was prepared by treatment of the isolated THBG sample with acetic anhydride. On reanalysis, using SRM conditions appropriate for the tetraacetate derivative, the shoulder on the peak disappeared (see Figure 24B). These data suggest that the interfering peak observed in the control urine was not THBG. The LC-SRM-MS chromatogram for the mouse-urine sample (see Figure 14B) revealed a much more distinct shoulder on the

side of the peak than was visible with the rat-urine sample (see Figure 13B). Furthermore, no interfering substances at the retention time of THBG were observed in the control mouse-urine sample (see Figure 14A).

A more complex LC gradient was employed to separate the shoulder on the peak observed for THBG in the mouse-urine sample. Again, no interference was observed in the control mouse-urine chromatogram under these new conditions (data not shown), but the two peaks in the THBG region were clearly separated for the urine from a mouse exposed to BD (see Figure 25A). The smaller, later eluting peak was shown to have an identical retention time to synthetic *meso*-THBG, which had been added to the sample (see Figure 25B), and so was identified as *meso*-THBG. The amount of *meso*-THBG corresponded to approximately 15% to 20% of the (±)-THBG in all of the samples examined. In light of these data, a small amount of the signal observed as a shoulder on the (±)-THBG in the rat-urine samples may have been derived from *meso*-THBG (see Figure 24A). Based on the size of the signal and the interference present in the control rat sample (see Figure 13A), however, the amount of *meso*-THBG was estimated to be less than 5% of the amount of (±)-THBG in each of the urine samples.

A series of rat- and mouse-liver DNA samples from animals exposed to 1,250 ppm BD was analyzed in order to determine whether any quantitative differences in DNA-adduct formation occurred between the species similar to those differences observed for urine. Interestingly, the same regioselectivity was observed with the BDO-derived N7-guanine adducts as had been observed in urine. Thus, 1HB2G was present in much greater amounts than was 2HB1G at every time point (see Figure 26). The relative amount of 2HB1G to 1HB2G was slightly higher in mice (approximately 0.2) than in rats (approximately 0.1). Concentrations of both 2HB1G and 1HB2G were lower in rat-liver DNA than in mouse-liver DNA (see Figure 26). The reduced concentrations of 2HB1G and 1HB2G in the rat-liver DNA reached statistical significance ($p < 0.05$) on all three days of exposure and for day 3 and day 6 after BD exposure. This contrasted with the postexposure urinary excretion data in which both of the N7-guanine BDO adducts were found in higher concentrations in the rat than in the mouse (see Figure 22).

From LC-MS analysis of THBG in hydrolyzed liver DNA of rats exposed to BD, it appeared that in addition to (±)-THBG, a significant amount of *meso*-THBG was present (see Figures 18B and 20B). This was confirmed by adding authentic *meso*-THBG to selected samples and identifying an increase in the signal with a retention time of 15.85 minutes observed in Figures 18B and 20B (data not shown). Further confirmation was made by acetylation of selected samples. As shown for a hydrolyzed liver-DNA sample from a rat exposed to BD, the retention times of (±)-THBG and *meso*-THBG (see Figure 28A) shifted when they were converted to tetraacetate derivatives (see Figure 28B). Although a fully validated assay for *meso*-THBG was not available, concentrations were determined from a standard curve using (±)-[$^{13}C_4$,^{15}N]THBG as the internal standard together with synthetic *meso*-THBG standards. Analyses of rat- and mouse-liver DNA samples for (±)-THBG and *meso*-THBG revealed a pattern of DNA-adduct formation similar to that observed for the BDO-derived N7-guanine derivatives. Less (±)-THBG was found in BD-exposed rat-liver DNA (see Figure 27A) than in BD-exposed mouse-liver DNA (see Figure 27B). The *meso*-THBG was present in slightly lower concentrations than was (±)-THBG at all time points in both BD-exposed rat-liver DNA (see Figure 27A) and BD-exposed mouse-liver DNA (see Figure 27B). Concentrations of (±)-THBG and *meso*-THBG in mouse-liver DNA were almost twice those observed in rat-liver DNA after 10 days of BD exposure, and the concentrations reached levels that were almost five times higher during the 6 days after exposure. A statistical analysis confirmed that the amounts of both (±)-THBG and *meso*-THBG were significantly lower ($p < 0.05$) during and after BD exposure in the liver DNA of rats compared with mouse-liver DNA (see Figure 27).

Regioselective formation and excretion of the BDO-guanine adducts 2HB1G and 1HB2G in vivo are difficult to reconcile with the in vitro data obtained with calf-thymus DNA and TK6-cell DNA. A similar (although smaller) regioselectivity was observed in a previous study using [^{32}P]-postlabeling: The ratio of 2HB1G to 1HB2G was 0.67 in the liver DNA of rats treated with a significantly lower dose of BD (200 ppm) (Koivisto et al. 1997). Conceivably, the 2HB1G was repaired from DNA more efficiently than was 1HB2G. This should have resulted in the excretion of more 2HB1G than 1HB2G in urine. However, urinary 1HB2G excretion was much greater than 2HB1G excretion in both rats and mice exposed to BD (see Figure 22). Examination of the structure of 2HB1G reveals that it is an allylic alcohol (see Figure 2). Therefore, 2HB1G could be a substrate for the aldehyde dehydrogenase, thought to be responsible for the formation of an intermediate enone during the formation of the urinary metabolite MI (see Figure 4) (Sabourin et al. 1992; Bechtold et al. 1994). This would result in formation of a metabolite that would not be detected by the LC-MS procedure. Alternatively, 2HB1G may undergo ring opening to form a derivative of 5-formamido-2,5,6-triaminopyrimidine in vivo similar to that observed for the AFB_1-guanine adduct (Hertzog et al.

1982). Again, this would result in the formation of a metabolite that would not be detected by LC-MS.

The observation that the THBG adduct is formed in vivo raises some interesting issues. First, the THBG found experimentally was a mixture of two diastereomers identical with the THBG derived from (±)-BDO_2 and *meso*-BDO_2, respectively. Although less (±)-THBG and *meso*-THBG was found in the liver DNA of rats exposed to BD compared with the liver DNA of mice exposed to BD, much more (±)-THBG was found than would have been predicted if the adducts had been derived solely from BDO_2. Previous studies have demonstrated that BDO_2 concentrations are almost undetectable in the blood of rats after exposure to BD, whereas significant concentrations of BDO_2 have been detected in the blood of mice exposed to BD. This finding suggests that some of the (±)-THBG and *meso*-THBG may have arisen from BDO-diol rather than from BDO_2 (see Figures 1 and 3). Alternatively, blood concentrations of BD-derived metabolites may not completely reflect metabolism in different organs and BDO_2 may be a significant metabolite in the rat. The present study raises the possibility that *meso*-BDO_2 is formed in vivo and that it may be an important determinant of carcinogenesis. In fact, in vitro metabolism studies have shown that *meso*-BDO_2 can be formed from BDO (Krause and Elfarra 1997). None of the in vivo BD-metabolism studies have had the specificity to distinguish between (±)-BDO_2 and *meso*-BDO_2, so no evidence has directly assessed their relative contributions to the overall BDO_2 concentrations.

As noted previously, the use of urinary N7-guanine adducts as biomarkers of exposure to electrophilic genotoxins is very attractive. The results of such studies have to be treated with some caution, however, because of the potential for formation of guanine adducts in the deoxynucleotide pool and in RNA with excretion of these adducts in urine. Previous studies with alkylating agents have demonstrated that guanine adducts are poorly repaired in RNA. In addition, guanosine adducts that would be present in RNA undergo spontaneous depurination at a much slower rate than do 2′-deoxyguanosine adducts present in DNA. For example, the half-life of RNA adducts is similar to that of RNA itself (namely, 5 days) (McElhon et al. 1971). Further, much less guanine is present in the deoxynucleotide pool than in DNA itself (Snow and Mitra 1987). Therefore, it is likely that a major component of the urinary N7-guanine adducts is derived from DNA rather than from RNA, the deoxynucleotide pool, or both. It was particularly interesting in the present study to find that mice (which are more susceptible to the carcinogenic effects of BD) had lower N7-guanine adduct concentrations in their urine than did the rats.

The apparent in vivo half-lives for 1HB2G and 2HB1G in rat-liver DNA were 3.5 days and 4.3 days, respectively. In mouse-liver DNA, the half-lives were almost the same, at 3.8 days and 4.3 days, respectively (see Figure 26). Similarly, the apparent in vivo half-lives observed for (±)-THBG and *meso*-THBG in rat-liver DNA were 3.6 days and 4.0 days, respectively (see Figure 27). However, the half-lives of (±)-THBG and *meso*-THBG appeared to be slightly greater in mouse-liver DNA, at 4.1 days and 5.5 days, respectively (see Figure 27). These half-lives are similar to those observed for N7-methyl–guanine and AFB_1-guanine (4 to 6 days), two compounds formed by spontaneous in vitro depurination of N7-guanine adducts (Lawley and Brooks 1963; Groopman et al. 1981). The greatest interspecies difference observed in the present study was the persistence of THBG in mouse liver (see Figure 27B) compared with that in rat liver (see Figure 27A). Thus, at day 6, almost five times as much (±)-THBG and *meso*-THBG was found in mouse liver as in rat liver. These data suggest that repair of both THBG adducts in the mouse is less efficient than in the rat. This hypothesis may help to explain the increased genotoxicity of BD in the mouse compared with genotoxicity in the rat.

SUMMARY AND CONCLUSIONS

The metabolism of BD to form DNA-reactive epoxides is extremely complex when the chirality of the products is considered (Figure 29). We have shown that BDO forms two regioisomeric N7-guanine adducts, each of which will exist as an enantiomeric pair, (*S*)- and (*R*)-2HB1G, and (*S*)- and (*R*)-1HB2G. Interestingly, both (±)-BDO_2 and *meso*-BDO_2 exclusively formed one regioisomeric N7-guanine adduct through nucleophilic attack at the terminal carbon atom of BDO_2. After depurination and hydrolysis, this resulted in the isolation of two N7-guanine adducts, (±)-THBG and *meso*-THBG, each of which will exist as a pair of two enantiomers (see Figure 29). Presumably, BDO-diol would show the same regioselectivity as BDO_2 to give the same N7-guanine adducts (see Figure 29). We cannot rule out the possibility, however, that an additional pair of regioisomers could be formed as a result of nucleophilic attack at C-2 of BDO-diol. Our LC-MS method was only able to separate 2HB1G from 1HB2G and (±)-THBG from *meso*-THBG. Therefore, we may have been unable to assess enantioselectivity in the formation of the epoxides mediated by cytochrome P450, in phase 2 metabolism of the epoxides, or in reaction of the epoxides with guanine bases in DNA.

There were more N7-guanine adducts of BDO in the liver DNA of mice than in the liver DNA of rats during the

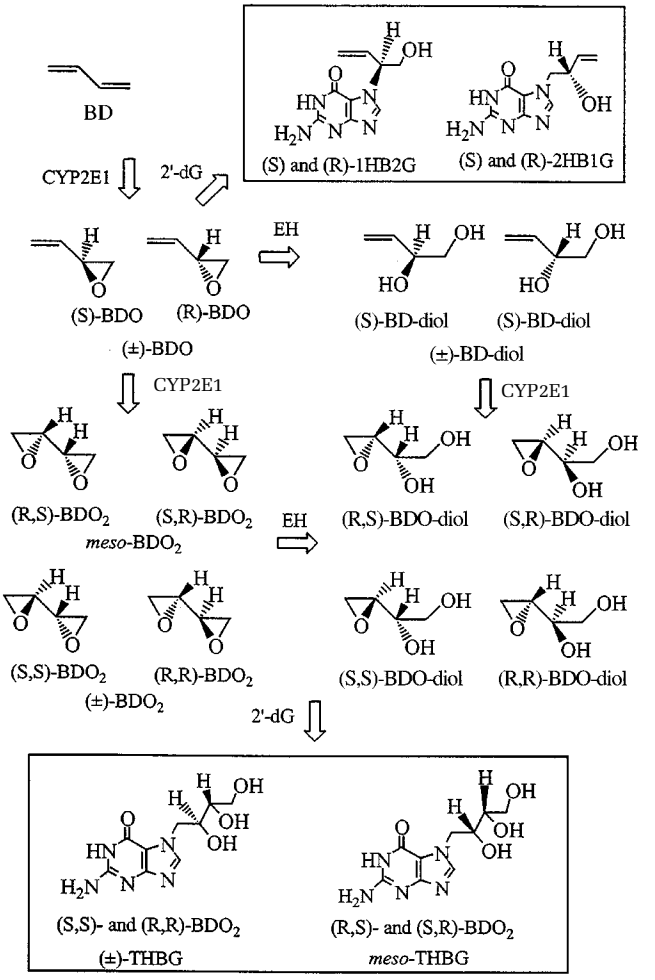

Figure 29. Cytochrome P450–mediated metabolism of BD and formation of N7-guanine adducts by reaction of chiral epoxide metabolites with 2′-deoxyguanosine (2′-dG). For simplicity, 1HB2G and 2HB1G are shown as the (*S*)-enantiomers, (±)-THBG is shown as the (*S*,*S*)-enantiomer, and *meso*-THBG is shown as the (*R*,*S*)-enantiomer. EH = epoxide hydroxylase.

10-day BD exposure and on day 3 and day 6 after exposure (see Figure 26). A regioselective difference was observed in the N7-guanine adducts derived from BDO that were present in the liver DNA of both rats and mice exposed to BD. The volume of 1HB2G exceeded 2HB1G by a factor of approximately 10 in the rat liver and by approximately 5 in the mouse liver (see Figure 26). This regioselective difference was apparent during the 10-day BD exposure and the 6 days after exposure. Because no regioselective difference was observed in the reaction between DNA and BDO in vitro, it was hypothesized that 2HB1G may be repaired from DNA more readily than 1HB2G. However, the half-lives of 2HB1G (4.3 days) and 1HB2G (3.5 days) in DNA were similar in rats and mice. Therefore, the observed regioselectivity must be a result of some other factor such as a structural modification of the 2HB1G. This could occur through ring opening to form a derivative of 5-formamido-2,5,6-triaminopyrimidine similar to that observed for the AFB_1-guanine adduct (Groopman et al. 1992). Alternatively, aldehyde dehydrogenase–mediated metabolism of 2HB1G (an allylic alcohol) to an enone may have occurred. This metabolic pathway is similar to that postulated for biotransformation of the allylic alcohol to an intermediate enone during formation of the urinary metabolite MI (see Figure 4) (Sabourin et al. 1992; Bechtold et al. 1994).

Higher amounts of 1HB2G appeared in rat urine than in mouse urine for all 3 days after BD exposure (see Figure 22). There was quantitatively much less 2HB1G than 1HB2G in both rat and mouse urine. However, the concentration of 2HB1G in rat urine was higher than in mouse urine. The increased urinary excretion in rats was difficult to reconcile with the similar half-lives of 2HB1G and 1HB2G in rat-liver DNA (4.1 and 3.5 days, respectively) and mouse-liver DNA (4.1 and 3.7 days, respectively).

The level of (±)-THBG was twice that of *meso*-THBG in the rat liver at all of the time points (see Figure 27A). After 10 days of exposure to BD, (±)-THBG was also present in an almost twofold excess over *meso*-THBG in the mouse liver (see Figure 27B). However, 6 days after exposure to BD, the two THBG diastereomers were present in almost equal amounts in the mouse liver. Furthermore, at this time point the amounts of the two THBG diastereomers in the mouse liver were almost fivefold greater than the amounts in the rat liver (see Figure 27). The half-lives of (±)-THBG and *meso*-THBG appeared to be longer in mouse-liver DNA (4.1 days and 5.5 days, respectively) than in rat-liver DNA (3.6 days and 4.0 days, respectively). Higher amounts of (±)-THBG appeared in rat urine than in mouse urine (see Figure 23). *meso*-THBG was detected in mouse urine, where it was found at a level of approximately 20% of that of (±)-THBG. The increased urinary excretion of (±)-THBG is in keeping with the apparently increased disappearance of (±)-THBG from the rat liver compared with levels in the mouse. This suggests that urinary (±)-THBG may be a useful index for helping to assess the relative risk of exposed human populations for BD-mediated carcinogenesis. The reduced urinary excretion of *meso*-THBG compared with excretion of (±)-THBG suggested that metabolism of *meso*-THBG may be occurring in vivo.

Data are scarce regarding the pharmacokinetic profiles of BDO-diol or *meso*-BDO_2 in rats and mice exposed to BDO-diol, and so it is difficult to assess the relative contributions of these metabolites to the formation of N7-guanine adducts. The present study has raised the possibility that *meso*-BDO_2 is formed in vivo and that it may be an important determinant of carcinogenesis (see Figure 29).

Surprisingly, all of the studies that have examined the mutagenic and carcinogenic potential of inhaled BDO_2 have employed only the commercially available (±)-BDO_2. The prevailing theory that BDO_2 is the major determinant of BD-induced carcinogenesis therefore requires further evaluation. From in vitro studies, *meso*-BDO_2 appears to be more toxic to hepatocytes than (±)-BDO_2 (IARC 1986). Therefore, it is conceivable that *meso*-BDO_2 may be more carcinogenic than (±)-BDO_2. Alternatively, the inherent toxicity of *meso*-BDO_2 may simply result in cell death, so that the transformation of cells to produce preneoplastic lesions cannot take place. Therefore, it will be important to determine whether *meso*-BDO_2 is mutagenic or carcinogenic and to assess its potency in comparison to that of (±)-BDO_2.

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REFERENCES

Adler ID, Filser JG, Gassner P, Kessler W, Kliesch U, Neuhauser-Klaus A, Nusse M. 1994. Mutagenicity of 1,3-butadiene inhalation in somatic and germinal cells of mice. Mutat Res 309: 307–314.

Albrecht OE, Filser JG, Neumann HD. 1993. Biological monitoring of 1,3-butadiene: Species differences in hemoglobin binding in rat and mouse. IARC Sci Publ 127:135–142.

Authrup H, Seremet T. 1986. Excretion of benzo[*a*]pyrene-guanine adduct in the urine of benzo[*a*]pyrene treated rats. Chem Biol Interact 60:217–226.

Autio K, Renzi, L, Catalan J, Albrecht OE, Sorsa M. 1994. Induction of micronuclei in peripheral blood and bone marrow erythrocytes of rats and mice exposed to 1,3-butadiene by inhalation. Mutat Res 309:315–320.

Bechtold WE, Shrunk MM, Chang I-Y, Ward JB Jr, Henderson RF. 1994. Species differences in urinary butadiene metabolites: Comparisons of metabolite ratios between mice, rats, and humans. Toxicol Appl Pharmacol 127:44–49.

Benner RA, Essigman JM, Wogan GN. 1981. Excretion of an aflatoxin-guanine adduct in the urine of aflatoxin B1 treated rats. Cancer Res 41:650–654.

Blair IA. 1993. Application of mass spectrometry to toxicology. Chem Res Toxicol 6:741–747.

Bolt HM, Jelitto B. 1996. Biological formation of the 1,3-butadiene DNA-adducts 7-N(2-hydroxy-3-buten-1-yl)guanine and 7-N-(1-hydroxy-3-buten-2-yl)guanine and 7-N-(2,3,4-trihydroxy-butyl)guanine. Toxicology 113:328–330.

Bond JA, Recio L, Andjelkovich D. 1995. Epidemiological and mechanistic data suggest that 1,3-butadiene will not be carcinogenic to humans at exposures likely to be encountered in the environment or workplace. Carcinogenesis 16:165–171.

Bonfanti M, Magagnotti C, Galli A, Bagnati R, Moret M, Gariboldi P, Fanelli R, Airoldi L. 1990. Determination of O^6-butylguanine in DNA by immunoaffinity extraction/gas chromatography–mass spectrometry. Cancer Res 50:6870–6875.

Brunnemann KD, Kagan MR, Cox JE, Hoffmann D. 1990. Analysis of 1,3-butadiene and other selected gas-phase components in cigarette mainstream and sidestream smoke by gas chromatography–mass selective detection. Carcinogenesis 11:1863–1868.

Budavari S, ed. 1998. 1,3-Butadiene. In: The Merck Index, 12^{th} ed. Merck & Co., Whitehouse Station, NJ.

Chapeau MC, Marnett LJ. 1991. Enzymatic synthesis of purine deoxynucleoside adducts. Chem Res Toxicol 4:636–638.

Chaudhary AK, Nokubo M, Marnett LJ, Blair IA. 1994. Analysis of malondialdehyde-2-deoxyguanosine adduct in rat liver DNA by gas chromatography/electron capture negative chemical ionization mass spectrometry. Biol Mass Spectrom 23:457–464.

Chaudhary AK, Nokubo M, Oglesby TD, Marnett LJ, Blair IA. 1995. Characterization of endogenous DNA adducts by liquid chromatography/electrospray ionization/tandem mass spectrometry. J Mass Spectrom 30:1157–1166.

Cheng X, Ruth JA. 1993. A simplified methodology for quantitation of butadiene metabolites: Application to the study of 1,3-butadiene metabolism by rat liver microsomes. Drug Metab Dispos 21:121–124.

Citti L, Gervasi G, Turchi G, Bellucci G, Bianchini R. 1984. The reaction of 3,4-epoxy-1-butene with deoxyguanosine

and DNA in vitro: Synthesis and characterization of the main adducts. Carcinogenesis 5:47–52.

Cochrane JE, Skopek TR. 1994. Mutagenicity of butadiene and its epoxide metabolites: II. Mutational spectra of butadiene, 1,2-epoxybutene and diepoxybutane at the *hprt* locus in splenic T cells from exposed B6C3F1 mice. Carcinogenesis 15:719–723.

Craddock VM, Magee PN. 1967. Effects of administration of the carcinogen dimethylnitrosamine on urinary 7-methylguanine. Biochem J 128:117–132.

Csanady GA, Guengerich FP, Bond JA. 1992. Comparison of the biotransformation of 1,3-butadiene and its metabolite, butadiene monoepoxide, by hepatic and pulmonary tissues from humans, rats and mice. Carcinogenesis 13:1143–1153.

Degan P, Shigenaga MK, Park EM, Alperin PE, Ames BN. 1991. Immunoaffinity isolation of urinary 8-hydroxy-2′-deoxyguanosine and 8-hydroxyguanine and quantification of 8-hydroxy-2′-deoxyguanosine in DNA by polyclonal antibodies. Carcinogenesis 12:865–871.

Divine BJ, Hartman CM. 1996. Mortality update of butadiene production workers. Toxicology 113:169.

Dizdaroglu M. 1986. Characterization of free radical-induced damage to DNA by the combined use of enzymatic hydrolysis and gas chromatography–mass spectrometry. J Chromatogr 367:357–366.

Djuric Z, Domenico AL, Luongo A, Harper DA. 1991. Quantitation of 5-(hydroxymethyl)uracil in DNA by gas chromatography with mass spectral detection. Chem Res Toxicol 4:687–691.

Duescher RJ, Elfarra AA. 1992. 1,3-Butadiene oxidation by human myeloperoxidase: Role of chloride ion in catalysis of divergent pathways. J Biol Chem 267:19859–19865.

Duescher RJ, Elfarra AA. 1994. Human liver microsomes are efficient catalysts of 1,3-butadiene oxidation: Evidence for major roles by cytochromes P450 2A6 and 2E1. Arch Biochem Biophys 311:343–349.

Fajen JM, Lunsford RA, Roberts DR. 1993. Industrial exposure to 1,3-butadiene in monomer, polymer and end-user industries. IARC Sci Publ 127:3–13.

Fajen JM, Roberts DR, Ungers LJ, Krishnan ER. 1990. Occupational exposure of workers to 1,3-butadiene. Environ Health Perspect 86:11–18.

Fenselau C. 1992. Tandem mass spectrometry: The competitive edge for pharmacology. In: Annual Review of Pharmacology and Toxicology (Cho AK, ed.) pp. 555–578. Annual Reviews, Inc, Palo Alto, CA.

Giese RW. 1997. Detection of DNA adducts by electron capture mass spectrometry. Chem Res Toxicol 10:255–270.

Goda Y, Marnett LJ. 1991. High-performance liquid chromatography with electrochemical detection for determination of the major malonaldehyde-guanine adduct. Chem Res Toxicol 4:520–524.

Goodrow T, Reynolds S, Maronpot R, Anderson M. 1990. Activation of K-ras by codon 13 mutations in C57BL/6 X C3H F1 mouse tumors induced by exposure to 1,3-butadiene. Cancer Res 50:4818–4823.

Greenblatt MS, Bennett WP, Hollstein M, Harris CC. 1994. Mutations in the p53 tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis. Cancer Res 54:4855–4878.

Groopman JD, Croy RG, Wogan GN. 1981. In vitro reactions of aflatoxin B1-adducted DNA. Proc Natl Acad Sci USA 78:5445–5449.

Groopman JD, Zhu J, Donahue PR, Pikul A, Zhang L, Chen J, Wogan GN. 1992. Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in Guagxi autonomous region, People's Republic of China. Cancer Res 52:45–52.

Harris CC. 1985. Future directions in the use of DNA adducts as internal dosimeters for monitoring human exposure to environmental mutagens and carcinogens. Environ Health Perspect 62:185–191.

Henderson RF, Bechtold WE, Bond JA, Sun JD. 1989. The use of biochemical markers in toxicology. Crit Rev Toxicol 20:65–82.

Hertzog PJ, Lindsay Smith JR, Garner R. 1982. Production of monoclonal antibodies to guanine imidazole ring opened aflatoxin B1-DNA, the persistent DNA adduct in vivo. Carcinogenesis 3:73–75.

Himmelstein MW, Acquavella JF, Recio L, Medinsky MA, Bond JA. 1997. Toxicology and epidemiology of 1,3-butadiene. Crit Rev Toxicol 27:1–108.

Himmelstein MW, Asgharian B, Bond JA. 1995. High concentrations of butadiene epoxides in livers and lungs of mice compared to rats exposed to 1,3-butadiene. Toxicol Appl Pharmacol 132:281–288.

Himmelstein MW, Turner MJ, Asgharian B, Bond JA. 1994. Comparison of blood concentratations of 1,3-butadiene and butadiene epoxides in mice and rats exposed to 1,3-butadiene by inhalation. Carcinogenesis 15:1479–1486.

Huff JE, Melnick RL, Solleveld HA, Haseman JK, Powers M, Miller RA. 1985. Multiple organ carcinogenicity of 1,3-butadiene in B6C3F1 mice after 60 weeks of inhalation exposure. Science 227:549.

International Agency for Research on Cancer. 1986. 1,3-Butadiene. In: Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. International Agency for Research on Cancer, Lyon, France.

International Institute of Synthetic Rubber Producers. 1981. The Toxicity and Carcinogenicity of Butadiene Gas Administered to Rats by Inhalation for Approximately 24 Months. Report No. 2653-522/2. International Institute of Synthetic Rubber Producers, Houston, TX.

Jajoo HK, Burcham PC, Goda Y, Blair IA, Marnett LJ. 1992. A thermospray liquid chromatography/mass spectrometry method for analysis of human urine for the major malondialdehyde-guanine adduct. Chem Res Toxicol 5:870–875.

Jelitto B, Vangala RR, Laib RJ. 1989. Species differences in DNA damage by butadiene: Role of diepoxybutane. Arch Toxicol Suppl 13:246–249.

Kim D-H, Guengerich FP. 1989. Excretion of the mercapturic acid S-[2-(N7-guanyl)ethyl]-N-acetylcysteine in urine following administration of ethylene dibromide to rats. Cancer Res 43:5077–5080.

Koivisto P, Sorsa M, Pacchierotti F, Peltonen K. 1997. ^{32}P-Postlabeling/HPLC assay reveals an enantioselective adduct formation in N7-guanine residues in vivo after 1,3-butadiene inhalation exposure. Carcinogenesis 18:439–443.

Krause RJ, Elfarra AA. 1997. Oxidation of butadiene monoxide to meso- and (±)-diepoxybutane by cDNA-expressed human cytochrome P450s and by mouse, rat, and human liver microsomes: Evidence for preferential hydration of meso-diepoxybutane in rat and human microsomes. Arch Biochem Biophys 337:176–184.

Lawley PD, Brooks P. 1963. Further studies on the the alkylation of nucleic acids and their constituent nucleotides. Biochem J 89:127–138.

Loeb LA. 1991. Mutator phenotype may be required for multistage carcinogenesis. Cancer Res 51:3075–3079.

McElhon MJ, O'Conner PJ, Craig AW. 1971. The stability of rat liver ribonucleic acid in vivo after methylation with methyl methanesulphonate or dimethylnitrosamine. Biochem J 125:821–827.

Melnick RL, Kohn MC. 1995. Mechanistic data indicate that 1,3-butadiene is a human carcinogen. Carcinogenesis 16:157–163.

Melnick RL, Huff J, Chou BJ, Miller RA. 1990. Carcinogenicity of 1,3-butadiene in C57BL/6 × C3H F1 mice at low exposure concentrations. Cancer Res 50:6592–6599.

National Toxicology Program. 1984. Toxicology and Carcinogenesis Studies of 1,3-Butadiene in B6C3F1 Mice. NTP TR 288, NIH Pub. No. 84-2544. US Public Health Service, US Department of Health and Human Services, Research Triangle Park, NC.

National Toxicology Program. 1993. Technical Report on the Toxicology and Carcinogenesis Study of 1,3-Butadiene. NTP TR 433. US Public Health Service, US Department of Health and Human Services, Research Triangle Park, NC.

Neagu I, Koivisto P, Neagu C, Kostiainen R, Stenby K, Peltonen K. 1995. Butadiene monoxide and deoxyguanosine alkylation products at the N^7-position. Carcinogenesis 16:1809–1813.

Neligan RE. 1962. Hydrocarbons in the Los Angeles atmosphere. Arch Environ Health 5:581.

Nieusma JL, Claffey DJ, Maniglier-Poulet C, Imiolczyk T, Ross D, Ruth JA. 1997. Stereochemical aspects of 1,3-butadiene metabolism and toxicity in rat and mouse liver microsomes and freshly isolated rat hepatocytes. Chem Res Toxicol 10:450–456.

Occupational Safety and Health Administration. 1990. Occupational exposure to 1,3-butadiene: Proposed rule. Fed Regist 55:33747.

Osterman-Golkar S, Bond JA, Ward JB Jr, Legator MS. 1993. The use of hemoglobin adducts as a tool for biomonitoring butadiene exposure. IARC Sci Publ 127:127–134.

Osterman-Golkar S, Ehrenberg L, Segerback D, Hallstrom I. 1976. Evaluation of genetic risks of alkylating agents: II. Haemoglobin as a dose monitor. Mutat Res 34:1–10.

Osterman-Golkar S, Peltonen K, Anttinen-Klemetti T, Landin HH, Zorcec V, Sorsa M. 1996. Haemoglobin adducts as biomarkers of occupational exposure to 1,3-butadiene. Mutagenesis 11:145–149.

Owen PE, Glaister JR, Gaunt IF, Pullinger DH. 1987. Inhalation toxicity studies with 1,3-butadiene: 3. Two-year toxicity/carcinogenicity studies in rats. Am Ind Hyg Assoc J 48:407–413.

Perera FP. 1988. The significance of DNA and protein adducts in human biomonitoring studies. Mutat Res 269:255–269.

Recio L, Osterman-Golkar S, Csanady GA, Turner MJ, Myhr B, Moss O, Bond JA. 1992. Determination of mutagenicity in tissues of transgenic mice following exposure to 1,3-butadiene and N-ethyl-N-nitrosourea. Toxicol Appl Pharmacol 117:58–64.

Reddy MV, Randerath K. 1986. Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. Carcinogenesis 7:1543–1551.

Reddy MV, Randerath K, Randerath E, Danna TF, Van Golen KL, Putman KL. 1989. A new sensitive ^{32}P-postlabeling assay based on the specific enzymatic conversion of bulky DNA lesions to radiolabeled dinucleotides and nucleoside 5′-monophosphates. Carcinogenesis 10:1231–1239.

Sabourin PJ, Burka LT, Bechtold WE, Dahl AR, Hoover MD, Chang IY, Henderson RF. 1992. Species differences in urinary butadiene metabolites: Identification of 1,2-dihydroxy-4-(N-acetylcysteinyl)butane, a novel metabolite of butadiene. Carcinogenesis 13:1633–1638.

Sangaiah R, Christova-Gueorguieva NI, Ranasinghe A, Tretyakova N-Yu, Gold A, Ball LM, Swenberg JA. 1997. Synthesis of perdeuterated analogues of the epoxide metabolites of butadiene: 1,2-Epoxybut-3-ene-d_6 and 1,2,3,4-diepoxybutane-d_6. J Label Compd Radiopharm 39:731–739.

Santella RM. 1988. Applications of new techniques for the detection of carcinogen adducts to human population monitoring. Mutat Res 205:271–282.

Santella RM, Yang XY, Hsieh LL, Young TL. 1990. Immunologic methods for the detection of carcinogen adducts in humans. Prog Clin Biol Res 340C:247–257.

Sathiakumar N, Delzell E, Hovinga M, Macaluso M, Julian JA, Larson R, Cole P, Muir DC. 1998. Mortality from cancer and other causes of death among synthetic rubber workers. Occup Environ Med 55:230–235.

Seaton MJ, Follansbee MH, Bond JA. 1995. Oxidation of 1,2-epoxy-3-butene to 1,2,3,4-diepoxybutane by cDNA-expressed human cytochromes P450 2E1 and 3A4 and human, mouse and rat liver microsomes. Carcinogenesis 16:2287–2293.

Selzer RR, Elfarra AA. 1996a. Characterization of N^1- and N^6-adenosine adducts and N^1-inosine adducts formed by the reaction of butadiene monoxide with adenosine: Evidence for the N^1-adenosine adducts as major initial products. Chem Res Toxicol 9:875–881.

Selzer RR, Elfarra AA. 1996b. Synthesis and biochemical characterization of N^1-, N^2-, and N^7-guanosine adducts of butadiene monoxide. Chem Res Toxicol 9:126–132.

Sharer JE, Duescher RJ, Elfarra AA. 1991. Formation, stability, and rearrangements of the glutathione conjugates of butadiene monoxide: Evidence for the formation of stable sulfurane intermediates. Chem Res Toxicol 4:430–436.

Shigenaga MK, Gimeno CJ, Ames BN. 1989. Urinary 8-hydroxy-2′-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. Proc Natl Acad Sci USA 86:9697–9701.

Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. 1994. Interindividual variations in human liver cytochromes P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: Studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 270:414–423.

Shuker DEG, Farmer PB. 1992. Relevance of urinary DNA adducts as markers of carcinogen exposure. Chem Res Toxicol 5:450–460.

Sisk SC, Pluta LJ, Bond JA, Recio L. 1994. Molecular analysis of *lacI* mutants from bone marrow of mice following inhalation exposure to 1,3-butadiene. Carcinogenesis 15:471–477.

Skipper PL, Tannenbaum S. 1990. Protein adducts in the molecular dosimetry of chemical carcinogens. Carcinogenesis 11:507–518.

Snow ET, Mitra S. 1987. Do carcinogen-modified deoxynucleotide precursors contribute to cellular mutagenesis? Cancer Invest 5:119–125.

Tates AD, van Dam FJ, de Zwart FA, van Teylingen CMM, Natarajan AT. 1994. Development of a cloning assay to detect induction of 6-thioguanine-resistant lymphocytes in spleen of adult mice following in vivo inhalation of 1,3-butadiene. Mutat Res 309:299–306.

Thornton-Manning JR, Dahl AR, Bechtold WE, Griffith WC Jr, Henderson RF. 1995. Disposition of butadiene monoepoxide and butadiene diepoxide in various tissues of rats and mice following a low-level inhalation exposure to 1,3-butadiene. Carcinogenesis 8:1723–1731.

Tretyakova NY, Chiang S-Y, Walker VE, Swenburg JA. 1998. Quantitative analysis of 1,3-butadiene-induced DNA adducts in vivo and in vitro using liquid chromatography

electrospray ionization tandem mass spectrometry. J Mass Spectrom 33:363–376.

Tretyakova NY, Lin Y-P, Sangaiah R, Upton PB, Swenberg JA. 1997a. Indentification and quantitation of DNA adducts from calf-thymus DNA exposed to 3,4-epoxy-1-butene. Carcinogenesis 18:137–147.

Tretyakova NY, Sangaiah R, Yen T-Y, Swenburg JA. 1997b. Synthesis, characterization, and in vitro quantitation of N7-guanine adducts of diepoxybutane. Chem Res Toxicol 10:779–785.

van Sittert J, van Vliet EWN. 1994. Monitoring exposure to some industrial chemicals by determining hemoglobin adducts. Clin Chem 40:1472–1475.

Weinberg RA. 1989. Oncogenes and the Molecular Origins of Cancer. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Weinstein IB. 1988. The origins of human cancer: Molecular mechanisms of carcinogenesis and their implications for cancer prevention and treatment. Cancer Res 48:4135–4143.

Weston A, Bowman ED. 1991. Fluorescence detection of benzo[*a*]pyrene-DNA adducts in human lung. Carcinogenesis 12:1445–1449.

Wild CP. 1990. Antibodies to DNA alkylation adducts as analytical tools in chemical carcinogenesis. Mutat Res 233:219–233.

Wolf SM, Vouros P. 1994. Application of capillary liquid chromatography coupled with tandem mass spectrometric methods to the rapid screening of adducts formed by the reaction of N-acetoxy-N-acetyl-2-aminofluorene with calf-thymus DNA. Chem Res Toxicol 7:82–88.

Yeola SN, Chaudhary AK, Marnett LJ, Blair IA. 1994. Preparation of isotope labeled [4,5,6,8-$^{13}C_4$; 9-$^{15}N_1$] guanine for the analysis of endogenous DNA adducts. In: Proceedings of the 42nd American Society of Mass Spectrometry Conference on Mass Spectrometry and Allied Topics, p. 367. American Society of Mass Spectrometry, Santa Fe, NM.

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PUBLICATIONS RESULTING FROM THIS RESEARCH

Kambouris SJ, Chaudhary AK, Selzer RR, Yeola SN, Elfarra AA, Recio L, Blair IA. 1996. Analysis of 1,2-epoxy-3-butene adducts of guanine in calf thymus DNA using liquid chromatography/electrospray ionization selected reaction monitoring tandem mass spectrometry (LC/ESI MS/MS). Proceedings of the 44th American Society of Mass Spectrometry Conference on Mass Spectrometry and Allied Topics, p. 315. American Society of Mass Spectrometry, Santa Fe, NM.

Kambouris SJ, Chaudhary AJ, Oe T, Blair IA. 1997. Quantitation of in vivo butadiene monoepoxide adducts of guanine using LC/API/MS/MS. Proceedings of the 45th American Society of Mass Spectrometry Conference on Mass Spectrometry and Allied Topics, p. 300. American Society of Mass Spectrometry, Santa Fe, NM.

Oe T, Chaudhary AK, Blair IA. 1997. LC/ESI MS/MS analysis of guanine-1,2,3,4-diepoxybutane adduct in urine of rats and mice exposed to 1,3-butadiene. Proceedings of the 45th American Society of Mass Spectrometry Conference on Mass Spectrometry and Allied Topics, p. 299. American Society of Mass Spectrometry, Santa Fe, NM.

Oe T, Kambouris SJ, Chaudhary AJ, Recio L, Blair IA. 1998. LC/API/SRM/MS analysis of guanine-1,2:3,4-diepoxybutane adduct in tissue of rats and mice exposed to 1,3-butadiene. Proceedings of the 46th American Society of Mass Spectrometry Conference on Mass Spectrometry and Allied Topics, p. 255. American Society of Mass Spectrometry, Santa Fe, NM.

Oe T, Kambouris SJ, Walker VE, Meng Q, Recio L, Wherli S, Chaudhary AK, Blair IA. 1999. Persistence of N7-(1,2:3,4-trihydroxy)guanine adducts in the livers of mice and rats exposed to 1,3-butadiene. Chem Res Toxicol 12:247-257.

ABBREVIATIONS AND OTHER TERMS

AcOH	acetic acid
AFB_1	aflatoxin B_1
ANOVA	analysis of variance
BD	1,3-butadiene
BD-diol	1,2-dihydroxy-3-butene
BDO	1,2-epoxy-3-butene
BDO_2	1,2,3,4-diepoxybutane
BDO-diol	1,2-dihydroxy-3,4-epoxybutane
CID	collision-induced dissociation
CYP2E1	cytochrome P450 2E1
D_2O	deuterated water
DMDO	dimethyldioxirane
D_6-DMSO	deuterated-dimethylsulfoxide
ESI	electrospray ionization
GC	gas chromatography
HCl	hydrochloric acid
^{1}H-NMR	proton nuclear magnetic resonance
1HB2G	N7-1-hydroxy-3-butenyl-2-guanine
2HB1G	N7-2-hydroxy-3-butenyl-1-guanine
HPLC	high-pressure liquid chromatography
HQC	higher concentration quality control
IARC	International Agency for Research on Cancer
IISRP	International Institute of Synthetic Rubber Producers
KOH	potassium hydroxide
LC	liquid chromatography
LLQ	lower limit of quantitation
LQC	lower concentration quality control
meso-THBG	diastereomer of THBG
MH^+	protonated molecular ion
MI	1,2-dihydroxy-4-(*N*-acetylcysteinyl)-butane
MII	1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene
MQC	middle concentration quality control
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
N_2	nitrogen
NH_4OAc	ammonium acetate
NH_4OH	ammonium hydroxide
NMR	nuclear magnetic resonance
NTP	National Toxicology Program
OSHA	Occupational Safety and Health Administration
PBA	phenylboronic acid
PBS	phosphate-buffered saline
Q1	first analyzer region of triple quadrupole
Q2	second (radio frequency only) analyzer region of triple quadrupole
Q3	third analyzer region of triple quadrupole

QC	quality control
(*R*)-BDO	stereoisomer of BDO
(*S*)-BDO	stereoisomer of BDO
SPE	solid-phase extraction
SRM	selected reaction monitoring
TFA	trifluoroacetic acid
THBG	N7-(2,3,4-trihydroxybutyl)guanine
(±)-THBG	diastereomer of THBG

1,3-Butadiene: Cancer, Mutations, and Adducts

Part V: Hemoglobin Adducts as Biomarkers of 1,3-Butadiene Exposure and Metabolism

James A. Swenberg, Nadia I. Christova-Gueorguieva, Patricia B. Upton, Asoka Ranasinghe, Nova Scheller, Kuen-Yuh Wu, Ten-Yang Yen, and Richard Hayes

ABSTRACT

1,3-Butadiene (BD)* is an important chemical used largely in the manufacture of synthetic rubber and thermoplastic resins. In addition, it has been identified in cigarette smoke, automobile exhaust, and gasoline vapor. The objective of this research was to develop highly sensitive and specific assays for the detection and quantitation of hemoglobin adducts of three BD metabolites: 1,2-epoxy-3-butene (BDO), 1,2,3,4-diepoxybutane (BDO_2), and 1,2-dihydroxy-3,4-epoxybutane (BDO-diol).

We have successfully developed an assay for both *N*-(2-hydroxy-3-butenyl)valine (HBVal) and *N*-(2,3,4-trihydroxybutyl)valine (THBVal) in hemoglobin. The six adducts measured were the two diastereomers (isomers I and II) of HBVal and the four diastereomers of THBVal (isomers I through IV, which were eluted as three peaks, 1, 2, and 3). HBVal and THBVal were measured in control and exposed $B6C3F_1$ mice and Sprague-Dawley rats (1,000 ppm BD for 13 weeks at 6 hours/day, 5 days/week). In a second set of animal exposures, total THBVal was determined in $B6C3F_1$ female mice (n = 5) exposed to 1,250 ppm BD for 1, 5, or 10 days (6 hours/day, 5 days/week). THBVal adducts were also monitored in occupationally exposed Chinese workers and nonoccupationally exposed U.S. laboratory workers.

This study utilized the modified Edman degradation method of Törnqvist and colleagues (1986). Briefly, the samples were subjected to Edman degradation, Centricon-30 ultrafiltration, washing on C_{18} columns, and acetylation for isomers of THBVal only, followed by gas chromatography–mass spectrometry (GC-MS) quantitation.

For the HBVal assay, an authentic internal standard globin alkylated with [$^2H_6$]BDO was used; for the THBVal assay, a synthesized external standard, THB[$^{13}C_5$]Val, was used after Edman degradation.

The mean ± SD amounts of total HBVal measured in exposed mice (in pmol/g globin) were 16,560 ± 3,910 for female mice (n = 4) and 12,400 ± 2,030 for male mice (n = 5). The corresponding values for rats were 8,690 ± 930 for female rats (n = 5) and 5,480 ± 2,880 for male rats (n = 3). The total amount of THBVal (eluted peaks 1, 2, and 3) in male mice (n = 5) was 78,900 ± 13,700; and in females (n = 2) was 56,100 ± 100. In male rats (n = 3), the detected value was 9,650 ± 1,620 and in females (n = 3) the value was 21,600 ± 6,780. In control male mice (n = 4), the total level of THBVal isomers was ~27 pmol/g globin. In a control male rat, total THBVal was ~15 pmol/g globin.

In the time course study, the amount of THBVal adducts increased linearly with exposure, resulting in values of 4,200 ± 830, 19,760 ± 1,780, and 35,940 ± 3,460 pmol/g globin following 1, 5, or 10 days of exposure to 1,250 ppm BD, respectively.

Detection of HBVal in human samples was difficult due to low concentrations of adducts and a high background in the chromatograms. In a pooled sample from 4 individuals, we performed multiple separations with high-pressure liquid chromatography (HPLC) of the derivatized adducts and detected 4.6 pmol/g globin (that is, 2.7 and 1.9 pmol/g globin for isomers I and II, respectively). We measured the amounts of THBVal in both

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

This Investigators' Report is Part V of Health Effects Institute Research Report Number 92, which also includes four additional Investigators' Reports (Parts I through IV) by different research groups, a Commentary by the Health Review Committee about Parts IV and V, a Commentary on Part I, a Commentary on Parts II and III, and an HEI Statement about the research projects reported here. Correspondence regarding the Part V Investigators' Report may be addressed to Dr. James A. Swenberg, Department of Environmental Sciences and Engineering, Curriculum in Toxicology, CB #7400, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

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nonoccupationally exposed U.S. laboratory workers and occupationally exposed workers from a polybutadiene plant in China. The mean total amount of THBVal among the U.S. laboratory workers was 36 ± 23 pmol/g globin for nonsmokers (n = 7) and 40 ± 9 for smokers (n = 4), compared with a mean total amount of 39 ± 13 pmol/g globin in a control set of Chinese workers (n = 25). These control values are overestimations of the true values because the amounts of THBVal in globin samples from other unexposed individuals (15 of 51) were below our limit of detection. BD-exposed Chinese workers had a total amount of 88 ± 59 pmol/g globin THBVal. The difference between smokers and nonsmokers was not significant, whereas the difference between control and exposed Chinese workers was highly significant ($p < 0.001$).

These data demonstrate that the methods developed in this project have adequate sensitivity to permit human biomonitoring for BD-hemoglobin adducts.

INTRODUCTION

1,3-Butadiene is an important industrial chemical used largely in the manufacture of synthetic rubber (namely, styrene-butadiene rubber and polybutadiene rubber) and thermoplastic resins (namely, acrylonitrile-butadiene-styrene). The annual production volume of BD in the United States is approximately 3 billion pounds, and worldwide annual production is approximately 12 billion pounds (Morrow 1990). BD has been identified in cigarette smoke, automobile exhaust, and gasoline vapor. Low levels of BD (0.5 to 10 ppb) have been detected in ambient air in some urban locations. Industrial production and use are estimated to account for 1.6% of environmental BD with mobile emissions representing 78.8% and miscellaneous sources accounting for 19.6% (U.S. Environmental Protection Agency 1994). Approximately 52,000 American workers are considered to be possibly exposed to BD (National Institute for Occupational Safety and Health 1990). A comprehensive review of BD toxicology and epidemiology was published by Himmelstein and colleagues (1997).

CARCINOGENICITY

1,3-Butadiene is carcinogenic in mice and rats following chronic inhalation exposure (National Toxicology Program 1984, 1993; Owen et al. 1987). Significant species differences exist in carcinogenic potency. Mice develop increased neoplasia following exposures to 6.25 ppm BD and higher. Rats are much less susceptible to BD-induced carcinogenesis, but they have been evaluated only at exposures of 1,000 or 8,000 ppm BD. The major differences in carcinogenic potency between rats and mice are associated with clear differences in metabolism (Himmelstein et al. 1997).

Until recently, epidemiologic studies have provided few consistent data on the carcinogenic risks of BD for humans (International Agency for Research on Cancer 1987). A comprehensive study on 15,649 BD workers by Delzell and colleagues (1996) has shown that exposures in the styrene-butadiene rubber industry cause higher incidences of leukemia with increasing exposure and duration of employment. In contrast, increases in leukemia incidence have not been shown in workers exposed to BD monomer. Instead, a few studies have found increased incidences of lymphosarcoma, but only in workers with short-term exposure. In these same cohorts, workers with exposure at the same time, but continuing for long term, did not show increases in the incidence of lymphosarcoma. Other individuals, including monomer workers in the Delzell study, did not have an excess in incidence of either lymphosarcoma or leukemia (Divine and Hartman 1996). Presently, the data support a conclusion that the styrene-butadiene rubber manufacturing process is carcinogenic for humans, but the evidence for BD monomer is limited.

METABOLISM

A simplified metabolic scheme for BD is shown in Figure 1. Three electrophilic metabolites are formed: BDO, BDO_2, and BDO-diol. BDO represents the initial metabolite formed by cytochrome P450 2E1 (*CYP2E1*) oxidation. BDO can undergo detoxification by glutathione or epoxide hydrolase. The latter reaction produces 1,2-dihydroxy-3-butene (BD-diol), which can be conjugated by glutathione to form the urinary metabolite 1,2-dihydroxy-4-(*N*-acetylcysteinyl)butane (MI) (Sabourin et al. 1992; Bechtold et al. 1994). BD-diol can undergo a second oxidation to form BDO-diol. Likewise, BDO can be further metabolized by *CYP2E1* to form BDO_2, which in turn can be hydrolyzed by epoxide hydrolase to BDO-diol. Several investigators have shown that mice produce much greater amounts of BDO_2 than do rats. Metabolism studies on BD using human liver suggest that humans are more like rats than mice in the type and amount of metabolites formed; however, considerable interindividual variation in humans was noted (Seaton et al. 1995; Boogaard and Bond 1996; Boogaard et al. 1996).

GENOTOXICITY

The genotoxic effects of BD are considered to be due to the reaction of its three major metabolites, BDO, BDO_2, and

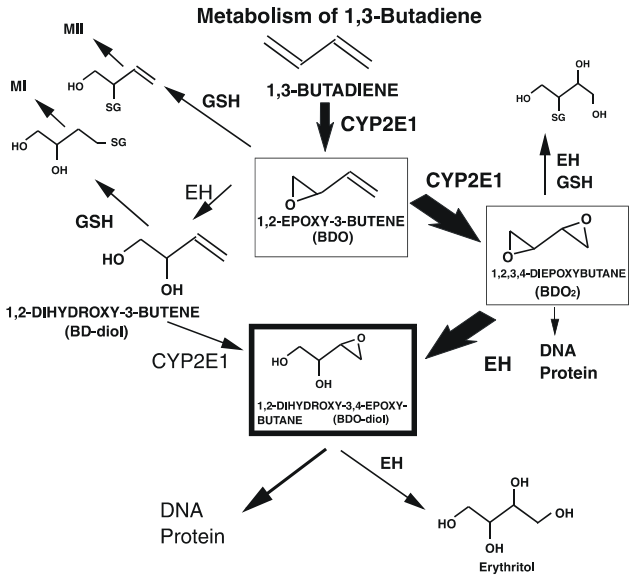

Figure 1. BD Metabolism. The three structures enclosed in boxes represent electrophiles that can covalently bind to the *N*-terminal valine of hemoglobin. MI = 1,2-dihydroxy-4-(*N*-acetylcysteinyl)butane, and MII = 1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene (both urinary metabolites of BD); GSH = reduced glutathione; and EH = epoxide hydroxylase.

BDO-diol, with the nucleophilic centers in DNA. BDO_2 is nearly 100 times more mutagenic in vitro in human TK6 lymphoblasts than is either BDO or BDO-diol (Cochrane and Skopek 1994). BDO_2 is also much more toxic than the other epoxides. Furthermore, nearly half of the mutations induced by BDO_2 are large deletions, whereas such deletions only occur at background frequencies in BDO-exposed cultures. BD is also genotoxic in vivo, inducing micronuclei in mice (Adler et al. 1994; Autio et al. 1994; Stephanou et al. 1997; Xiao et al. 1996), but not in rats (Autio et al. 1994). It also induces heritable translocations in mice (Adler et al. 1995, 1998) and *hprt* mutations in rats and mice (Tates et al. 1994, 1998; Meng et al. 1996). BD has produced variable results in humans (Tates et al. 1996; Legator et al. 1993; Ward et al. 1994) and genetic effects in germ cells in mice (Brinkworth et al. 1998).

GENETIC POLYMORPHISMS

Several investigators have demonstrated that the null genotype for the polymorphisms in the glutathione *S*–transferase *T1* (*GSTT1*) and *M1* (*GSTM1*) loci confers increased genotoxic susceptibility to human lymphocytes exposed to BDO_2 and BDO, respectively (Wiencke et al. 1995; Uusküla et al. 1995; Norppa et al. 1995). It is also reasonable to expect that polymorphisms in the epoxide hydrolase and *CYP2E1* loci could affect the rates of detoxification and activation of BD and its metabolites.

DNA ADDUCTS

The identification and quantitation of DNA adducts formed by BD and its metabolites have been actively studied by several research teams during the past five years. DNA adducts have been characterized at the N7 position of guanine and the N1, N3, and N6 positions of adenine (Citti et al. 1984; Leuratti et al. 1994; Neagu et al. 1995; Tretyakova et al. 1996, 1997a,b,c, 1998; Koivisto et al. 1996, 1998). Multiple DNA adducts are formed at each of the above positions because attack at the C1 and C2 positions of BDO and at the C1 position of both BDO-diol and BDO_2 are possible. This is further complicated by diastereomers if nucleosides or nucleotides are being measured as well as by stereoisomers of the prime 2′,3′,4′-(trihydroxybut-1′-yl)-adenine and -guanine adducts formed by BDO-diol and racemic and *meso*-BDO_2.

HEMOGLOBIN ADDUCTS

Measurements of hemoglobin adducts offer some important advantages over other measurements of internal dose, including the following: (a) Blood samples are readily available for epidemiologic and biomonitoring studies and contain large amounts of hemoglobin. (b) The analysis of protein adducts is highly sensitive and specific. Hemoglobin adducts are not repaired, and they thus accumulate for the lifespan of the erythrocyte, allowing studies to be carried out at low exposure levels. (c) Studies of hemoglobin adducts in humans can provide considerably improved exposure assessments because they integrate exposure over time. (d) Adducts provide a measure of the dose of reactive chemicals in laboratory animals and in humans, thereby eliminating some of the difficulties in extrapolating from high to low doses and across species (Osterman-Golkar et al. 1993).

The BD metabolite BDO has been shown to react with hemoglobin to form HBVal adducts (Osterman-Golkar et al. 1991, 1993). Two major and two minor peaks were identified with use of GC-MS. The two major peaks were shown to be the diastereomers resulting from attack on the *N*-terminal valine-NH_2 at the C1 position of BDO. Adduct concentrations of 1 to 3 pmol/g globin were recorded in nonsmoking persons working in a production area with ~1 ppm BD levels. Adducts also were measured in cigarette smokers who were not occupationally exposed to BD. The reported adduct levels were lower in humans than in mice and rats exposed to 2 ppm BD and were also lower than hydroxyethylvaline adducts associated with

occupational exposures to ethylene oxide and ethylene. Albrecht and colleagues (1993) reported adduct amounts five times higher in mice than in rats (17 and 3.5 nmol/g globin, respectively, after exposure to 500 ppm, 6 hours/day, for 5 days), although the diastereomers were not resolved. It is clear that BD exposure results in a supralinear dose response that is characteristic of saturation of metabolic activation and that mice have higher amounts of monoepoxide adducts than do rats (Albrecht et al. 1993; La and Swenberg 1997). This conclusion is also supported by two studies on the induction of micronuclei in rats and mice (Adler et al. 1994; Autio et al. 1994). In pilot studies, we compared male and female rats and mice exposed to 1,000 ppm BD for 13 weeks and found that females had higher levels of BDO-hemoglobin adducts than did males (Tretyakova et al. 1996). All of the hemoglobin studies have utilized the modified Edman degradation method of Törnqvist and colleagues (1986), which is based on GC-MS measurements using an internal standard of [$^2H_4$]-*N*-(2-hydroxyethyl)valine ([$^2H_4$]HEVal), [^{14}C]-*N*-(2-hydroxypropyl)valine, or *N*-(2-hydroxy-butenyl)valine-glycine-glycine.

At the time this project was initiated, there were no reports of measurements of the hemoglobin adducts of BDO-diol or BDO_2. In view of the greater formation of BDO_2 by the mouse compared with formation in the rat, and the much greater mutagenicity of BDO_2, it was important that methods be developed so that both quantitative and relative comparisons of hemoglobin adducts could be made between species. During the conduct of this project, Pérez and colleagues (1997) reported formation of THBVal adducts in hemoglobin of rats and humans. The THBVal adducts were formed in greater amounts than the previously measured levels of HBVal adducts. The authors concluded that BDO-diol appeared to be an important metabolite of BD.

SPECIFIC AIMS

The objective of this research was to develop high- resolution mass spectrometric methods for quantitating the hemoglobin adducts of BD and to utilize these methods to analyze globin samples from mice, rats, and humans exposed to BD. The long-term goals of this research were to better understand the mechanisms of BD carcinogenicity and to improve the accuracy of cancer risk assessment for BD. The adducts studied include those formed by BDO, BDO_2, and BDO-diol with the *N*-terminal valine of globin.

METHODS AND STUDY DESIGN

This research project was designed to develop new or improved methods for measuring the number and type of hemoglobin adducts in blood from laboratory animals and humans using GC-MS techniques.

SPECIMENS AVAILABLE FOR ANALYSIS

Washed red blood cell (RBC) samples were available for species comparisons between rats and mice. These samples had been stored at –70°C for 5 years and had been obtained from male and female Crl:CD®BR rats and $B6C3F_1$/CrlBR mice exposed by inhalation to 1,000 ppm BD (6 hours/day, 5 days/week, for 13 weeks) at the Haskell Laboratory for Toxicology and Industrial Medicine, E. I. duPont de Nemours and Company (Newark, DE). The animals had been fed Certified Purina Laboratory Chow #5002 and had water available ad libitum. These samples were expected to have steady-state levels of HBVal and THBVal adducts because the exposure period exceeded the life span of the erythrocytes. An additional set of mouse blood samples (which had been stored for 1 year) was available from $B6C3F_1$ female mice exposed to 1,250 ppm for 1, 5, or 10 days (6 hours/day, 5 days/week) at the Chemical Industry Institute of Toxicology (Research Triangle Park, NC). These animals were fed Lab-Blox chow and had water ad libitum except during exposures. Based on published data regarding BD metabolism and hemoglobin and DNA adducts, the 1,250 ppm concentration of BD saturates the metabolism of BD to its epoxide intermediates. Therefore, the 13-week exposure samples should have the highest number of BD adducts possible.

For human studies, we had 87 coded samples of RBCs from workers in a polybutadiene plant in China. The set included samples from both exposed and unexposed individuals and constituted a small portion of the analyses conducted in a large molecular epidemiologic study (Hayes et al. 1996). As such, the samples provided molecular dosimetry data on hemoglobin adducts to compare with exposure data, *hprt*$^-$ mutations, and other measured biomarkers. Institutional Review Board approval was obtained at the time the specimens were collected; we were exempt from the need for further approval because we had no information that identified the sampled individuals. After analysis of THBVal adducts, the data were sent to Dr. Hayes for decoding and comparing with exposure measurements. In addition, the number of THBVal adducts in humans were compared with those found in BD-exposed rats and mice. The study had limited ability to demonstrate whether or not large interindividual differences in adduction exist because only limited exposure

data were available. Finally, the study provided information on the limits of detection of hemoglobin adducts that will be useful for designing future molecular epidemiologic studies of occupational and environmental exposure. During the evaluation of samples from Chinese workers, we obtained 13 additional blinded human samples from laboratory volunteers at the University of North Carolina to compare with the samples obtained in China.

CHEMICALS

BD (99% pure) and racemic mixtures of BDO (98% pure) and BDO_2 (97% pure) were purchased from Aldrich (Milwaukee, WI). [$^2H_6$]BD (98% enriched) was obtained from Cambridge Isotopes (Andover, MA). Pentafluorophenylisothiocyanate (PFPITC) and formamide were purchased from Fluka (Buchs, Switzerland). The [$^2H_4$]HEVal globin standard was provided by John MacNeela from the Chemical Industry Institute of Toxicology. HBVal, HB[$^{13}C_5$]Val, THBVal, and THB[$^{13}C_5$]Val were synthesized by Dr. J. Krzeminski from American Health Foundation under contract from the National Cancer Institute. All other reagents and solvents used were analytic-reagent or HPLC grade.

BLOOD SAMPLE STORAGE

Samples of saline-washed RBCs from rats, mice, and humans were frozen in liquid nitrogen and stored at −70°C until analyzed. This period of time ranged from 1 to 5 years for rodent samples and was 3 years for the Chinese human samples.

ISOLATION OF GLOBIN

Frozen washed RBCs from mice, rats, and humans were thawed and diluted in equal volumes of distilled deionized water (DDW). Globin was isolated according to the method of Mowrer and colleagues (1986), as modified slightly by Walker and colleagues (1993) for mouse globin. DDW (1 mL) was added to 1 mL of washed packed RBCs, and the tubes were inverted gently to ensure complete mixing. After the mixing was complete, the solution was allowed to sit for several minutes, and then 50 mM HCl in 2-propanol was added (6 mL for rat globin, 9 mL for mouse globin, and 8 mL for human globin). The tubes were mixed thoroughly, employing a vortex at a setting of 2 or 3, and then centrifuged at 4°C at 1,500 × *g* for 30 minutes. The supernatant was carefully decanted into a fresh tube, and 4 mL ethyl acetate was added for rat samples, 6 mL for mouse samples, and 5 mL for human samples. The pellet was discarded. The ethyl acetate-supernatant mixture was vortexed thoroughly until the solution turned cloudy with globin precipitate. The samples were centrifuged at 4°C at 1,500 × *g* for 5 minutes, the supernatant discarded, and the globin pellet saved. Ethyl acetate (4 mL) was added to resuspend the pellet. The tube was centrifuged at 4°C at 1,500 × *g* for 5 minutes and the supernatant discarded. This step was repeated until the supernatant was colorless. Then the pellet was resuspended in 4 mL of *n*-pentane, centrifuged, and the supernatant discarded. The final pellet was dried carefully under a gentle stream of nitrogen gas. After the pellet was dry, the pellet was transferred to a clean, labeled screw-cap vial. The vials (loosely capped) were placed in a vacuum desiccator under house vacuum for several hours or overnight, and then they were placed under a stronger vacuum for several more hours. The samples were then derivatized or stored in vials (tightly capped) at − 70°C.

DERIVATIZATION OF GLOBIN SAMPLES

All glassware used for sample derivatization and cleanup was silanized in a 5% solution of dimethyldichlorosilane in *n*-pentane, rinsed twice with methanol, and air dried. The derivatization was performed according to the *N*-alkyl Edman procedure, based on Törnqvist's modified Edman degradation for specific cleavage of *N*-alkylated terminal valines of the four chains in hemoglobin (Törnqvist 1986). Routinely, 2 to 5 mg mouse globin, 6 to 10 mg rat globin, or 50 to 250 mg human globin was dissolved in 1.5 mL formamide (volume for up to 50 mg globin). A few further modifications to the method included the addition of 20 µL 1M NaOH/mL formamide to each sample and the addition of 10 µL PFPITC/50 mg globin to the human samples. All samples were shaken overnight (about 18 hours) at room temperature (25°C) and then for 1.5 hours at 45°C. The samples were filtered through Centricon-30 columns, the retentate was discarded and the filtrate used.

HBVal Adducts

An internal standard of rat globin treated with [$^2H_6$]BDO (see Standards section) and containing 48 pmol *N*-terminal [$^2H_6$]HBVal/mg globin (total for both diastereomers) was added to each sample. After completion of the Edman degradation, the samples were extracted three times with 2 mL diethyl ether, dried under nitrogen gas, and then redissolved in toluene. The samples were washed: first with 2 mL DDW, next with 2 mL freshly prepared 0.1 M sodium carbonate (Na_2CO_3), and then with 2 mL DDW. The toluene layer was evaporated under nitrogen gas, and the samples were then reconstituted in 50 µL dry toluene for GC-MS analysis.

Method I for THBVal Adducts

For rodent samples, 5 to 15 mg globin was used, whereas 100 to 150 mg globin was used for all human samples. After completion of the Edman degradation, an external standard of THB[$^{13}C_5$]Val-pentafluorothiohydantoin (PFPTH) was added, and samples were filtered through Centricon-30 columns. The rodent samples were extracted three times with 3 mL ether, dried under nitrogen gas, and then redissolved in toluene and washed with 1 mL 0.1 M Na_2CO_3 followed by 1 mL DDW to minimize loss due to analyte solubility in aqueous solutions. For the human samples, after evaporating the pooled ether extractions, the residue was dissolved in 200 µL formamide and applied to prepared 500-mg C_{18} columns (50:50 formamide:DDW), and washed sequentially with 1 mL each of DDW, 0.1 M Na_2CO_3, and DDW. After drying the columns, the samples were eluted in 4 mL dry acetonitrile and evaporated under nitrogen gas. The dry samples were acetylated with 25% triethylamine and 25% acetic anhydride in acetonitrile for 30 minutes at room temperature (25°C), dried, resuspended in *n*-pentane, and washed with 3 mL 60% aqueous methanol. The *n*-pentane layer was evaporated and resuspended in 20 to 50 µL toluene for GC-MS analysis. The samples were stable and could be stored for several months at –20°C.

Method II for THBVal Adducts

For rodent samples, 5 to 15 mg globin was used; for all human samples, 100 to 150 mg globin was used. After completion of the Edman degradation, an external THB[$^{13}C_5$]Val-PFPTH standard was added, and samples were filtered through Centricon-30 columns. The samples were extracted three times with 3 mL diethyl ether and dried under nitrogen gas. The residue was dissolved in 1 mL fresh 0.1 M Na_2CO_3 and applied to prepared 100-mg C_{18} columns (50:50 formamide:DDW), and washed with 1 mL DDW. After drying the columns, the samples were eluted in 3 mL dry acetonitrile and evaporated under nitrogen gas. The dry samples were acetylated with 25% triethylamine and 25% acetic anhydride in acetonitrile for 30 minutes at room temperature (25°C), dried, resuspended in 3 mL *n*-pentane, and washed with 2 mL 60% aqueous methanol. The *n*-pentane layer was evaporated and resuspended in 20 to 50 µL toluene for GC-MS analysis. The samples were stable and could be stored for several months at –20°C.

LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY ANALYSIS

The technique of liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) (negative mode) was conducted on synthesized standards using a Pharmacia liquid chromatographic system with dual pumps (#2248, Pharmacia LKB Biotechnology, Uppsala, Sweden) coupled to a Finnigan 4000 quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) that was retrofitted with a pneumatic electrospray source (Analytica of Branford, Branford, CT). The LC column used was C_{18} (150 mm × 0.8 mm internal diameter; Hypersil, 3 µm particle size, LC Packings, San Francisco, CA). The flow rate was 22 µL/minute. The LC separation was performed by the following program: 50% to 100% aqueous methanol for 10 minutes, 100% from 10 to 20 minutes, and 100% to 50% from 20 to 35 minutes.

GAS CHROMATOGRAPHY–MASS SPECTROMETRY ANALYSIS

Samples were analyzed by GC-MS in the electron-capture negative chemical ionization (ECNCI) mode with methane as reagent gas (3×10^{-5} mbar) and using a VG 70-250SEQ hybrid mass spectrometer with a Hewlett-Packard 5890 GC. The GC separation was performed on an Alltech, SE-54 column (30 mm × 0.32 mm, 1.0 µm film thickness; Alltech Associates, Deerfield, IL) with 10 psi He head pressure and 300°C injector temperature. Injections were made in the direct mode with the following column temperature program: 100°C to 300°C at the rate of 10°C per minute. The instrument was operated at 70 eV and emission current at 500 µA. The source temperature was ~170°C and 250°C during HBVal and THBVal analysis, respectively. Full-scan mass spectra were obtained at a resolving power of 1,000. Quantitative selected ion monitoring (SIM) measurements were performed at a resolving power of 10,000. The ions monitored were at a mass-to-charge ratio (*m*/*z*) of 318.0450 and 320.0573 (for HBVal) and 534.1084 and 539.1254 (for THBVal) for the analyte and the external standard, respectively. Three replicates were analyzed from each rodent sample. Duplicate samples were analyzed from humans if enough globin was available. Perfluorokerosene was used for tuning and calibrating the mass spectrometer (Ranasinghe et al. 1998).

STANDARDS

To generate an internal standard of [$^2H_6$]HBVal globin, the following procedure was used: A solution containing 20 mg [$^2H_6$]BDO in 7 mL acetone synthesized and quantitated according to Sangaiah and colleagues (1997) was added to 2 mL freshly washed and lysed RBCs. The mixture was incubated at 37°C for 3 hours and the globin was isolated according the method of Mowrer and colleagues (1986).

The four standards of HBVal-PFPTH, HB[$^{13}C_5$]Val-PFPTH, THBVal-PFPTH, and THB[$^{13}C_5$]Val-PFPTH were prepared from adducted valine standards synthesized by Dr. Krzeminski from the American Health Foundation. Synthesis was conducted according to the procedure of Törnqvist (1994) and had more than 90% yield. The HBVal-PFPTH and HB[$^{13}C_5$]Val-PFPTH standards were prepared by dissolving HBVal or HB[$^{13}C_5$]Val in 3 mL 0.5 M potassium bicarbonate ($KHCO_3$) and 1.5 mL 1-propanol; 10 µL PFPITC (pH 8) was added, and the solution was incubated for 3 hours at 45°C. This was extracted two times with 2 mL heptane and evaporated under nitrogen gas. The THBVal-PFPTH and THB[$^{13}C_5$]Val-PFPTH standards were prepared by dissolving THBVal or THB[$^{13}C_5$]Val in 1 mL 0.5 M $KHCO_3$ and 0.5 mL 1-propanol; 10 µL PFPITC (pH 8) was added, and the solution was incubated for 2 hours at 45°C, after which 1.0 mL DDW was added. This was extracted four times with 3 mL ether, evaporated under nitrogen gas, dissolved in 3 mL toluene, washed in 1 mL 0.1 M Na_2CO_3 and 1 mL DDW, and dried again under nitrogen gas.

The isomers of the analytes and internal standards were dissolved in formamide and separated by HPLC using UV detection (268 nm). For primary separation, a semipreparative column (Beckman Ultrasphere C_{18}, 25 cm × 0.60 cm, 5 µm) was used with a linear gradient of 10% to 100% methanol in water over 20 minutes. The fractions of interest from multiple injections were pooled together and further purified on an analytical column (Ultrasphere C_{18}, 25 cm × 0.46 cm, 5 µm) using the same mobile-phase and gradient program. All separated isomers were quantitated by UV absorption at 268 nm. The standards were further characterized by GC-MS in ECNCI mode and by LC-ESI-MS full-scan spectra.

CALIBRATION

The calibration solutions were prepared by dissolving the standards in 1.5 mL formamide containing control rat globin (10 mg per sample) as a protein carrier. In the case of HBVal, we added 0.45 mg internal standard globin with [$^2H_6$]HBVal and different amounts of globin with HBVal, which were obtained by in vitro alkylation of RBCs with unlabeled BDO as described previously. Then the solutions were processed using the same protocol as for sample preparation. Two separate calibration curves were built for both diastereomers. The synthesized HBVal-PFPTH and HB[$^{13}C_5$]Val-PFPTH (latter added after the Edman degradation step) were also used as reference standards for stability control of the internal standard [$^2H_6$]HBVal globin.

In the case of THBVal, 1.5 mL formamide samples containing control rat globin (10 mg per sample) as a protein carrier were subjected to Edman degradation. Different amounts of the THBVal-PFPTH standard and 8 pmol of THB[$^{13}C_5$]Val-PFPTH external standard were added to the samples, and the calibration solutions were processed by the same protocol as for sample preparation. Separate calibration curves were built for the three diastereomer peaks of THBVal as measured by GC-MS.

QUALITY ASSURANCE

The laboratory worked with the intent of Good Laboratory Practices. Each of our staff was required to keep a laboratory notebook acceptable to Good Laboratory Practices standards. These notebooks were archived in a central location when full and not in use. Samples were stored in a freezer at −70°C until processed. The GC-MS instrumentation was carefully tuned and calibrated before use; each calibration was noted and a known solution similar to the samples was run to check for day-to-day reproducibility. A synthesized derivatized standard was run every fifth sample to ensure run-to-run reproducibility and stability of calibration.

STATISTICAL ANALYSES

The coefficient of variation for assay performance was determined using one-way analysis of variance (ANOVA). Comparisons of adduct concentrations between (1) male and female rats, (2) male and female mice, (3) male mice and male rats, (4) female mice and female rats, and (5) all mice and all rats were made by ANOVA followed by the Tukey-Kramer multiple-comparisons test. A value of $p < 0.05$ was required for statistical significance. Comparisons of THBVal between BD-exposed and unexposed controls in the Chinese human molecular epidemiologic study were made by the t test, as was the comparison between THBVal values of smokers and nonsmokers. A value of $p < 0.05$ was required for statistical significance. Least-squares regression analysis was used to compare THBVal and measurements of environmental exposure. Values are reported as means ± SD.

RESULTS

MASS SPECTROMETRY

Standards

The synthesized standards HBVal-PFPTH and THBVal-PFPTH, as well as the corresponding [$^{13}C_5$]-labeled external standards, were separated by HPLC as described above

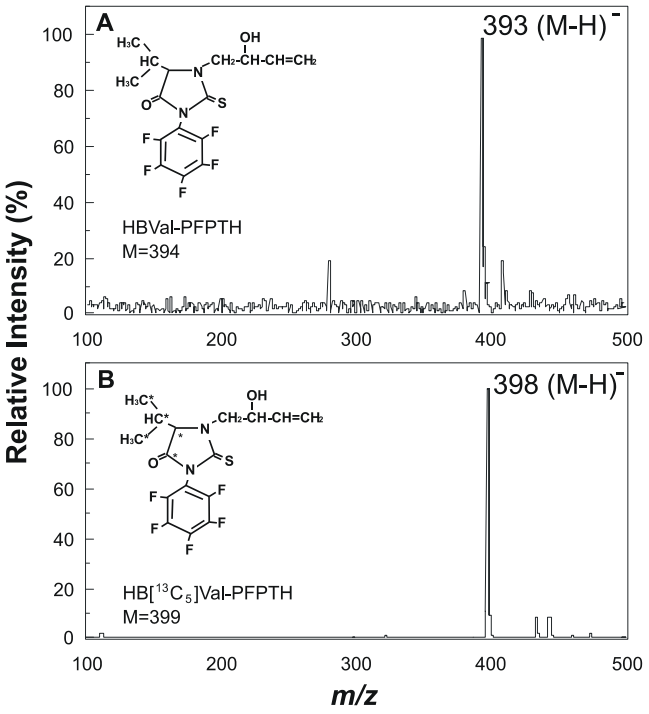

Figure 2. LC-ESI-MS (negative mode) spectra of (A) HBVal-PFPTH and (B) HB[$^{13}C_5$]Val-PFPTH.

in the Standards section. Figure 2 illustrates the LC-ESI-MS (negative mode) spectra for isomer I of HBVal-PFPTH (Figure 2A) and HB[$^{13}C_5$]Val-PFPTH (Figure 2B), demonstrating the most abundant ions $(M–H)^-$ at *m/z* 393 and 398 for the analyte and external standard, respectively. A similar full scan could not be run on the [$^2H_6$]HBVal globin. Figure 3 represents full-scan GC-ECNCI-MS spectra of isomer I of HBVal-PFPTH (Figure 3A), [$^2H_6$]HBVal globin (Figure 3B), and HB[$^{13}C_5$]Val-PFPTH (Figure 3C). There were no differences in the fragmentation patterns in the full-scan MS spectra for the diastereomers. The main fragment ions in the GC-ECNCI-MS spectra for HBVal-PFPTH were *m/z* 374 [M–HF] and *m/z* 318 [M–CH_2═CH–CHO]. The corresponding ions for the deuterated internal globin standard were *m/z* 380 and *m/z* 320, whereas those for the [$^{13}C_5$] external standard were at *m/z* 379 and *m/z* 323. Under our conditions, the highest sensitivity was achieved by SIM of the ions at *m/z* 323, 320, and 318. We attempted tandem mass spectrometry (MS/MS) of *m/z* 374 to 318 (*m/z* 380 to 320, respectively, for the deuterated internal globin standard), but the sensitivity was about 20 times less compared with direct SIM of [M–HF], although the background level was substantially decreased.

The LC-ESI-MS (negative mode) spectra for THBVal-PFPTH (Figure 4A) and the [$^{13}C_5$]-labeled external standard

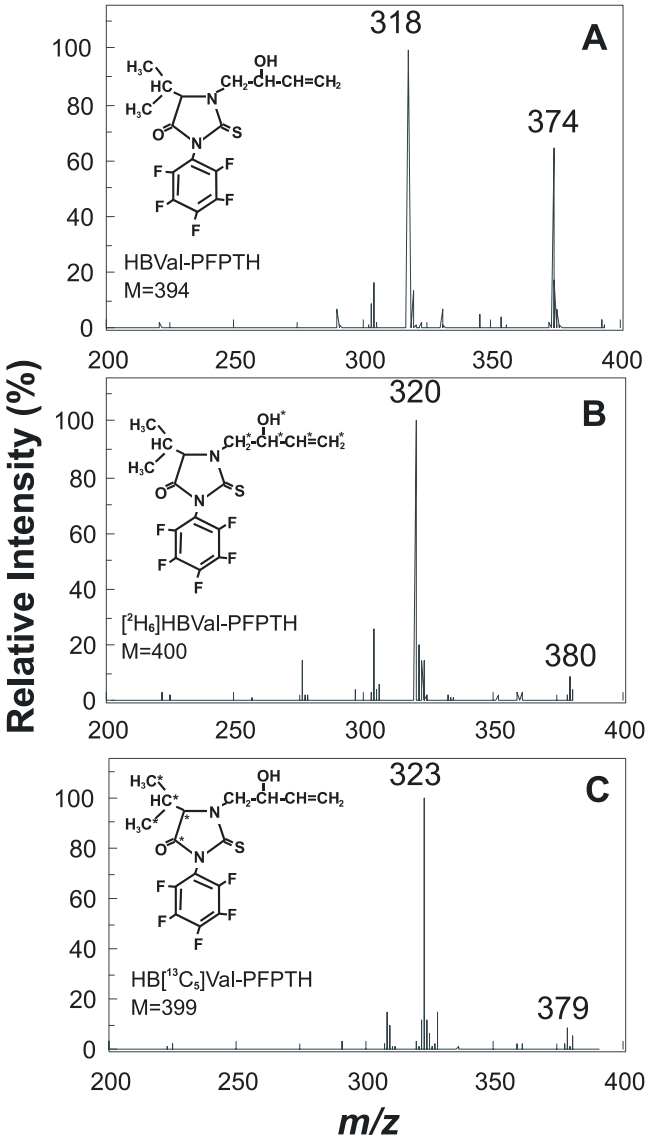

Figure 3. Full-scan GC-ECNCI-MS spectra of (A) HBVal-PFPTH, (B) [$^2H_6$]-HBVal globin, and (C) HB[$^{13}C_5$]Val-PFPTH.

(Figure 4B) demonstrate $(M–H)^-$ peaks at *m/z* 427 and 432, re- spectively. The full-scan GC-ECNCI-MS spectra of THBVal-PFPTH (after acetylation) and THB[$^{13}C_5$]Val-PFPTH (peak 3, after acetylation) are shown in Figure 5. For the GC-MS analysis of THBVal-PFPTH and THB-[$^{13}C_5$]Val-PFPTH, the dominant fragment ions were *m/z* 534 and 539, respectively, corresponding to [M–HF]. The isotopic labeling pattern of the ions helps to elucidate the fragmentation mechanism of the molecular ions during ECNCI processes. The common ion, *m/z* 451, is due to the loss of the valine group and HF (M–HF–C_5H_7O). The ions *m/z* 534 and *m/z* 323 (Figure 5A) originate from the loss of HF and the acetylated butadiene group [$C_4H_6(OAc)_3$],

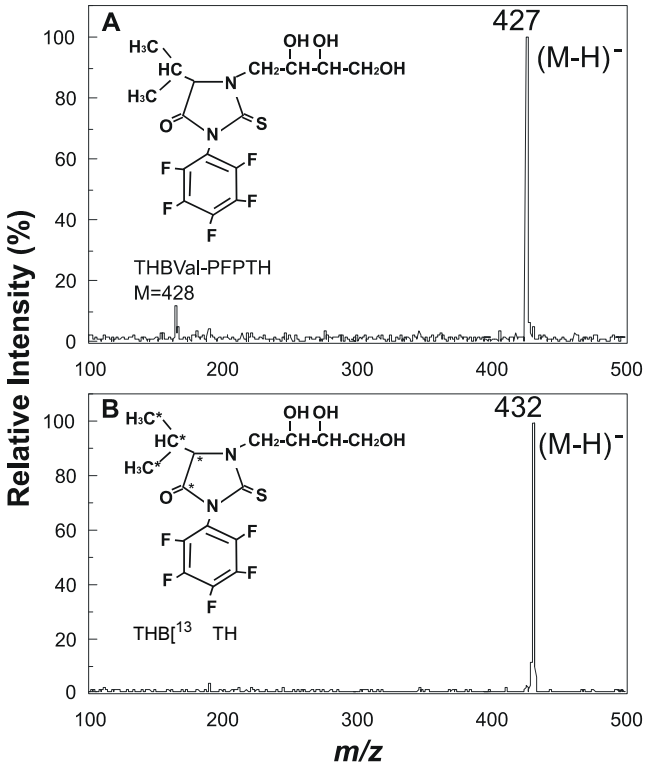

Figure 4. LC-ESI-MS (negative mode) spectra of (A) THBVal-PFPTH and (B) THB[$^{13}C_5$]Val-PFPTH.

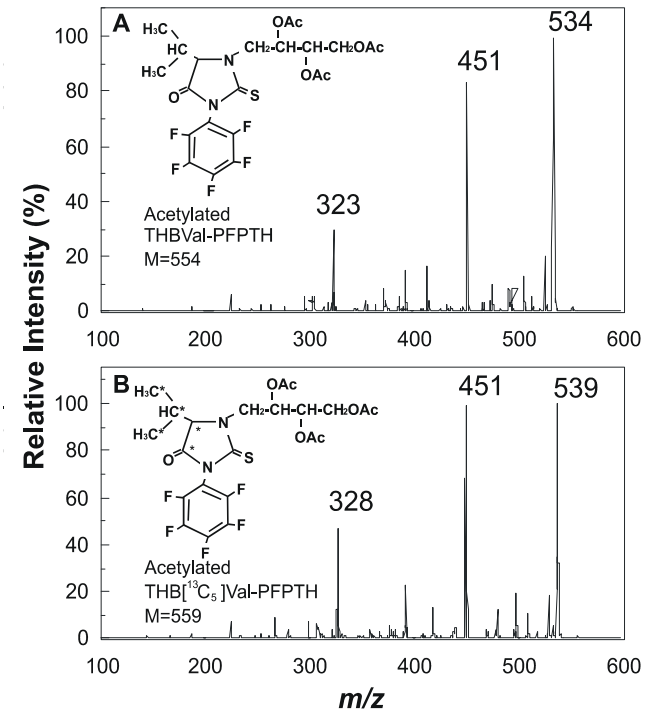

Figure 5. Full-scan GC-ECNCI-MS spectra of (A) THBVal-PFPTH and (B) THB[$^{13}C_5$]Val-PFPTH.

respectively. This is confirmed by the presence of all of the ^{13}C labels, as indicated by the 5 amu mass shifts in the corresponding fragment ions, *m/z* 539 and 328 (Figure 5B). There were relatively low backgrounds in the THBVal chromatograms at these SIMs.

Calibration Curves

The calibration curves for both isomers (namely, I and II) of HBVal globin versus the internal standard [$^2H_6$]- HBVal globin are shown in Figure 6. The corresponding calibration curves for THBVal-PFPTH versus THB[$^{13}C_5$]Val-PFPTH (diastereomer peaks 1, 2, and 3) are presented in Figure 7. All calibration curves gave a linear response in the concentration range used for quantitation.

PERFORMANCE OF VARIOUS METHODS

During the course of methods development and sample analysis, it became apparent that both the HBVal globin standard and the corresponding [$^2H_6$]HBVal globin internal standard degraded when stored at –20°C, but not at –70°C. To establish the amount of [$^2H_6$]HBVal in the alkylated globin, it was compared with an [$^2H_4$]HEVal globin standard containing 16.3 pmol/mg globin. Three independent experiments, analyzing 5 to 6 replicates each, were performed, and the values of 27.65 ± 2.04 pmol/mg globin for isomer I and 20.36 ± 1.83 pmol/mg globin for isomer II of [$^2H_6$]HBVal were obtained. To control for standard degradation, synthesized HBVal-PFPTH and its [$^{13}C_5$]-labeled standard were also used to check globin standard concentrations. The reported HBVal measurements were based on a deuterated internal globin standard that had been calibrated using standard curves constructed with [$^2H_4$]HEVal.

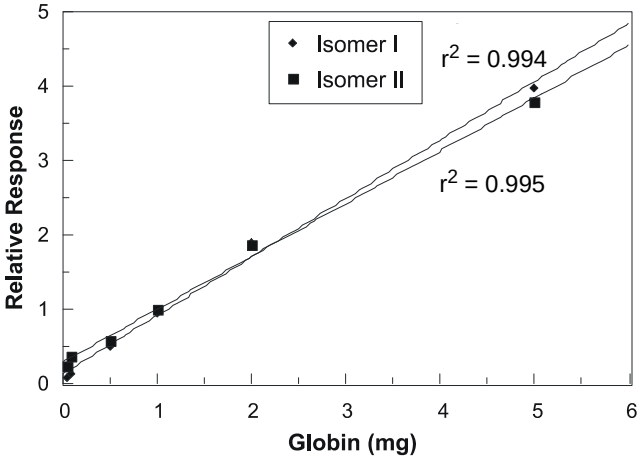

Figure 6. Calibration curves for HBVal-PFPTH (isomers I and II) versus [$^2H_6$]HBVal-PFPTH.

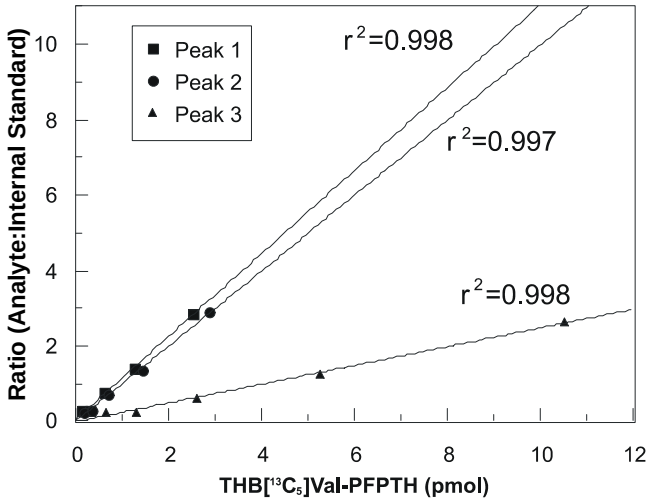

Figure 7. Calibration curves for THBVal-PFPTH (peaks 1, 2, and 3) versus THB[$^{13}C_5$]Val-PFPTH.

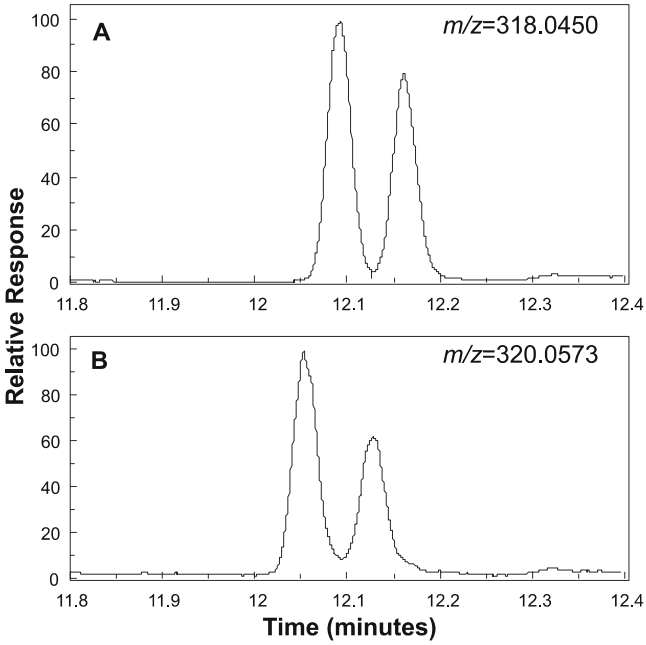

Figure 8. GC-ECNCI-MS chromatograms of (A) HBVal in a globin sample from a male rat exposed to 1,000 ppm BD for 13 weeks (9 mg globin) and of (B) the internal standard, [$^2H_6$]HBVal globin. In each chromatogram, isomer I is the first peak and isomer II is the second, smaller peak.

For quantitation of THBVal in the rodent and human samples noted in this report, the [$^{13}C_5$]-labeled external standard for THBVal was added after the Edman degradation because this reaction is considered to be very reproducible and of high yield (Törnqvist 1986; Bergmark 1997). THBVal adducts in globin appeared to be stable when stored at −70°C. Samples of rat and human globin were analyzed by different individuals more than a year apart with good agreement in analysis results.

The recovery of analytes after sample preparation was monitored by adding, at the end of the procedure, synthesized standards HBVal-PFPTH or THBVal-PFPTH to calibration solutions that contained known amounts of both analyte and internal standard. The total recovery was established to be 10% to 12% in both assays. Most of the loss occurred during the clean-up steps.

Reproducibility of the method for total HBVal was evaluated using triplicate globin samples (including two isomers) from 17 animals from the 13-week, 1,000 ppm BD exposure. The coefficient of variation was ± 9%; however, interindividual variability was greater. Reproducibility of the assay for total THBVal was established by repeated processing of 8 rodent globin samples from the 13-week, 1,000 ppm BD exposure. These latter samples were processed up to 16 months apart by two different individuals. The coefficient of variation for these assays was ± 14%.

ANALYSIS OF HBVal AND THBVal IN RATS AND MICE EXPOSED TO BD

A representative HBVal GC-ECNCI-MS chromatogram of a globin sample from a rat exposed to 1,000 ppm BD for 13 weeks is shown in Figure 8. The chromatogram shows two HBVal peaks (isomers I and II, resulting from attack at the C1 position) with slightly different retention times for the analyte and the deuterated internal globin standard. The ratio between the intensity of the two peaks varied from 1.2 to 1.6. The natural isotopic contribution of nondeuterated HBVal to the deuterated internal standard (2%) was taken into account and the corresponding corrections made when necessary.

The data for HBVal adducts in mice and rats exposed to 1,000 ppm BD for 13 weeks (6 hours/day, 5 days/week) revealed about twice as many adducts in mice than in rats ($p < 0.001$; Table 1). Although females of both rodent species appeared to have higher numbers of HBVal adducts than males, the difference did not reach statistical significance.

The GC-ECNCI-MS chromatogram of THBVal from an exposed rat (1,000 ppm BD, 13 weeks at 6 hours/day, 5 days/week) is presented in Figure 9, together with a chromatogram of the THB[$^{13}C_5$]Val-PFPTH external standard. The three peaks correspond to the diastereomeric THBVal generated by BDO-diol and BDO_2. Peaks 1 and 2

Table 1. HBVal from Red Blood Cells from Mice and Rats Exposed to 1,000 ppm BD[a]

Animals (*n*)	Isomer I	Isomer II	Total
Mice			
Males (5)	7,020 ± 1,240	5,430 ± 790	12,400 ± 2,030
Females (4)	9,600 ± 2,070	6,970 ± 1,930	16,560 ± 3,910
Rats			
Males (3)	3,150 ± 1,560	2,340 ± 1,320	5,480 ± 2,880
Females (5)	4,990 ± 560	3,700 ± 420	8,690 ± 930

[a] Exposures were 6 hours/day, 5 days/week, for 13 weeks. Values are in pmol HBVal/g globin, and expressed as means ± SD.

correspond to the first peak published by Pérez and colleagues (1997), whereas peak 3 corresponds to the second peak monitored by the same authors.

The data for THBVal measured in exposed mice and rats are presented in Table 2. The concentration of THBVal adducts in female mice was ~2.5 times greater than that in female rats ($p < 0.05$), whereas male mice had ~8 times more THBVal than did male rats ($p < 0.001$). Likewise, THBVal was greater in all mice than in all rats ($p < 0.001$). Two comparisons, of male rats with female rats and male mice with female mice, showed no significant differences.

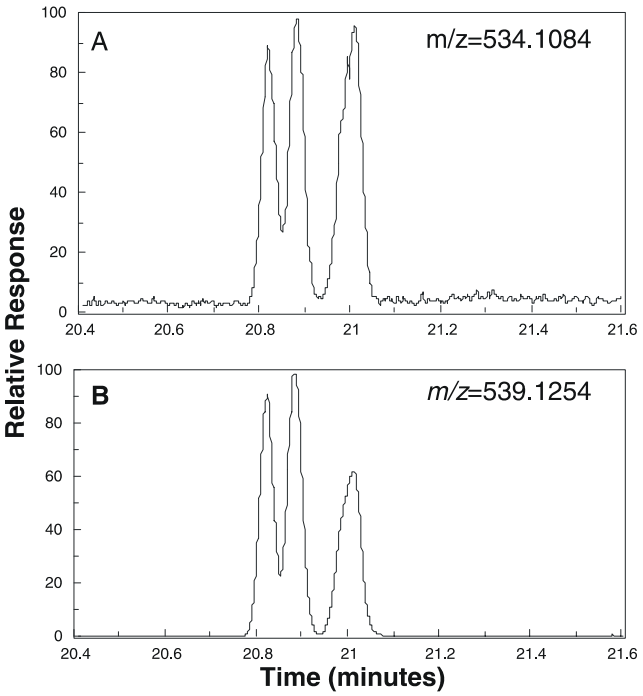

Figure 9. GC-ECNCI-MS chromatograms of (A) THBVal in a globin sample from a male rat exposed to 1,000 ppm BD for 13 weeks (6 mg globin) and of (B) the external standard, THB[$^{13}C_5$]Val-PFPTH. In each chromatogram, the first peak is isomer I, the second peak is isomer II, and the third peak contains isomers III and IV.

Table 2. THBVal from Red Blood Cells from Mice and Rats Exposed to 1,000 ppm BD[a]

Animals (*n*)	THBVal
Mice	
Males (5)	78,900 ± 13,700
Females (2)	56,100 ± 100
Rats	
Males (3)	9,650 ± 1,620
Females (3)	21,600 ± 6,780

[a] Exposures were 6 hours/day, 5 days/week, for 13 weeks. Values are in pmol THBVal/g globin, and expressed as means ± SD.

The THBVal adducts were also detectable in samples from a control male rat (15 pmol/g) and a pooled sample from four male mice (27 pmol/g), although much larger amounts of globin were needed for these analyses (80 to 120 mg). Thus, male mice and rats exposed to 1,000 ppm BD for 13 weeks had THBVal adducts that were ~3,000- and ~650-fold greater than levels in controls, respectively. When the data from Tables 1 and 2 are compared, the ratio of total THBVal to HBVal was 6.3 for male mice, 3.4 for female mice, 1.8 for male rats, and 2.5 for female rats. In all cases where THBVal and HBVal data were collected on the same animals, THBVal levels were greater.

Globin samples from a second study with female mice exposed to 1,250 ppm BD for 1, 5, or 10 days were analyzed for total THBVal (Figure 10). A linear increase in THBVal was associated with increased BD exposure. Comparing these data with the above data on endogenous THBVal in male mice suggests that a single 6-hour exposure to 1,250 ppm BD results in a increase of ~150-fold in THBVal adducts.

ANALYSIS OF HUMAN SAMPLES

The initial analyses of human samples concentrated on HBVal measurements in samples obtained from the molecular epidemiologic study of Hayes and colleagues (1996). Analysis of human globin samples for HBVal used 15 to 50 mg globin and followed the standard protocol for sample preparation. This work failed to detect peaks of the analyte in any sample.

Unfortunately, repeated attempts to measure HBVal depleted eight of the samples from Chinese workers. There were two main reasons for the difficulty in detecting HBVal: The concentration of adducts in the samples was too low, below our detection limit, and the background of the chromatograms was too high, with a large interfering peak present just in front of the expected analyte peaks. In order to decrease the background level,

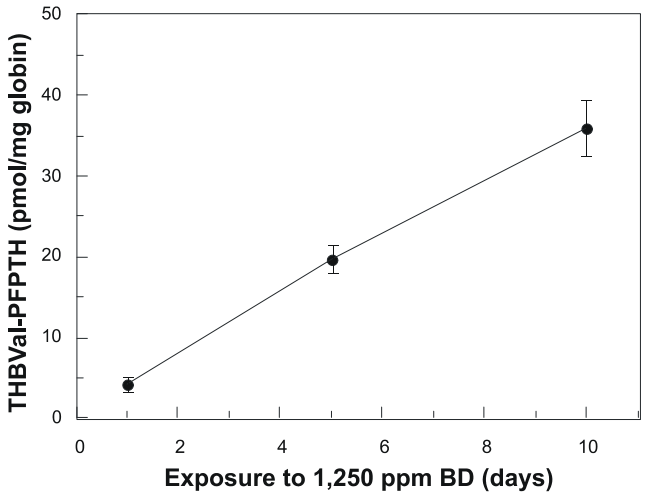

Figure 10. Relation between THBVal levels in female mice and duration of exposure to 1,250 ppm BD.

we tried to perform MS/MS (*m*/*z* 374 to 318). In one sample (54 mg globin) from a male worker in the recovery group, the peaks in the chromatogram were low, but after a smoothing operation we estimated the amount of HBVal to be 1.1 pmol/g globin for isomer I. The peak for isomer II was almost at the background level so no estimate could be made. This result demonstrated that MS/MS could lower the background. However, even for standards, the sensitivity of the MS/MS method was about 20 times lower than that of SIM high-resolution MS of *m*/*z* 318 and *m*/*z* 320 ions. The major reason for such poor sensitivity with MS/MS using this instrumentation appeared to be the extensive fragmentation that occurred in the source due to high temperature. This has not been a problem with other instruments (Ostermann-Golkar et al. 1991, 1993). A second attempt to measure HBVal used about 100 mg of pooled globin from four Chinese workers whose exposure groups were unknown to us. We performed multiple HPLC separations on the derivatized product using the same conditions as for standards (see Methods and Study Design). The process took several days, but we succeeded in monitoring HBVal at 2.8 and 1.9 pmol/g globin for isomers I and II, respectively, with large signal-to-noise ratios (Figure 11).

In contrast to HBVal, THBVal adducts were detectable in most human samples, including those from Chinese workers and from American volunteers (the latter including smokers and nonsmokers). The number of samples that had amounts of THBVal below our detection limit was greatest in the nonexposed (control) Chinese workers. Measurements could not be made in 6 of 24 female controls and 7 of 14 male controls. In contrast, only 8 of 41 workers had undetectable amounts of THBVal.

Figure 12 presents a representative chromatogram of a human globin sample of a nonexposed nonsmoker. Among samples from nonoccupationally exposed volunteers

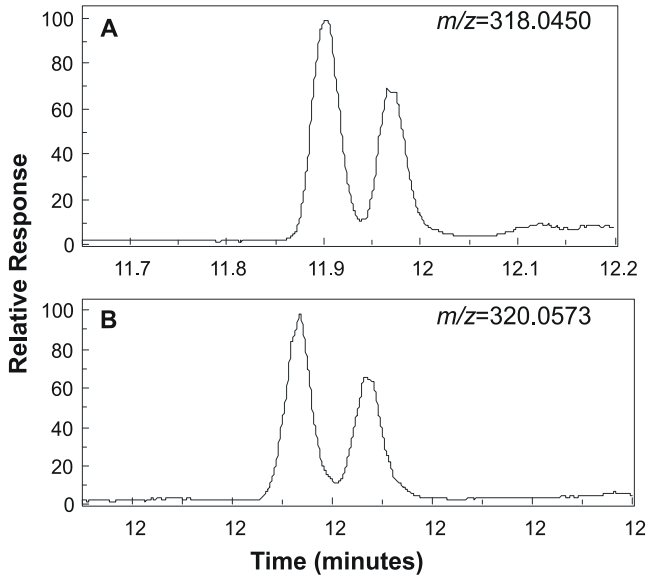

Figure 11. GC-ECNCI-MS chromatograms of (A) HBVal in a pooled blood sample from four Chinese workers and of (B) the internal standard, [$^2H_6$]-HBVal globin. Note that the peaks are similar to those shown in Figure 8.

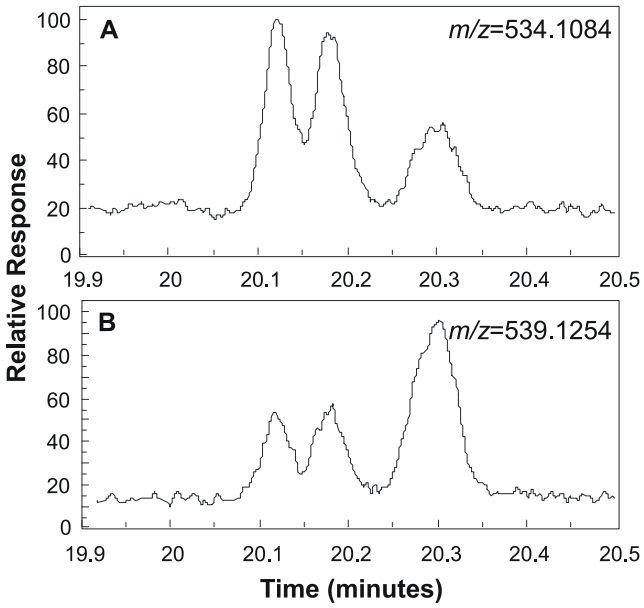

Figure 12. GC-ECNCI-MS chromatograms of (A) THBVal in a globin sample from an unexposed human (150 mg globin) and of (B) the external standard, THB[$^{13}C_5$]Val-PFPTH. In each chromatogram, the first peak is isomer I, the second peak is isomer II, and the third peak contains isomers III and IV.

(7 nonsmokers and 4 smokers), 11 of 13 samples had amounts of THBVal that were above our detection limit. The mean values for THBVal were 36 ± 23 pmol/g for nonsmokers and 40 ± 8 pmol/g for smokers. The difference between smokers and nonsmokers was not significant.

In the study of Chinese workers, we first analyzed the concentration of THBVal adducts in all individuals with or without potential occupational exposure to BD. The mean total amount of THBVal in 25 nonexposed workers was 39 ± 13 pmol/g globin, whereas the mean for 33 exposed workers was 88 ± 59 pmol/g globin. This difference was significant ($p < 0.001$). Male workers without occupational exposure to BD had values of 46 ± 20 pmol/g globin ($n = 7$), and female workers without occupational exposure had 37 ± 8 pmol/g ($n = 18$). The difference was not statistically significant. None of the females smoked, whereas 6 of the 7 males were smokers. The amount of THBVal in unexposed Chinese workers was not different from that of U.S. volunteers.

The THBVal data were then analyzed by job category (Hayes et al. 1996). Process analysts, whose job is to take BD samples from production process lines in either the polymerization or dimethyl formamide (DMF) facilities and then analyzed them by gas chromatography, had THBVal levels of 71 ± 24 ($n = 13$) and 140 ± 94 ($n = 7$) pmol/g globin, respectively. Figure 13 shows a chromatogram of THBVal adducts in globin from one of the DMF workers. Workers who did process control, minor maintenance, and when needed, major repairs had THBVal levels of 78 ± 48 ($n = 13$) pmol/g globin.

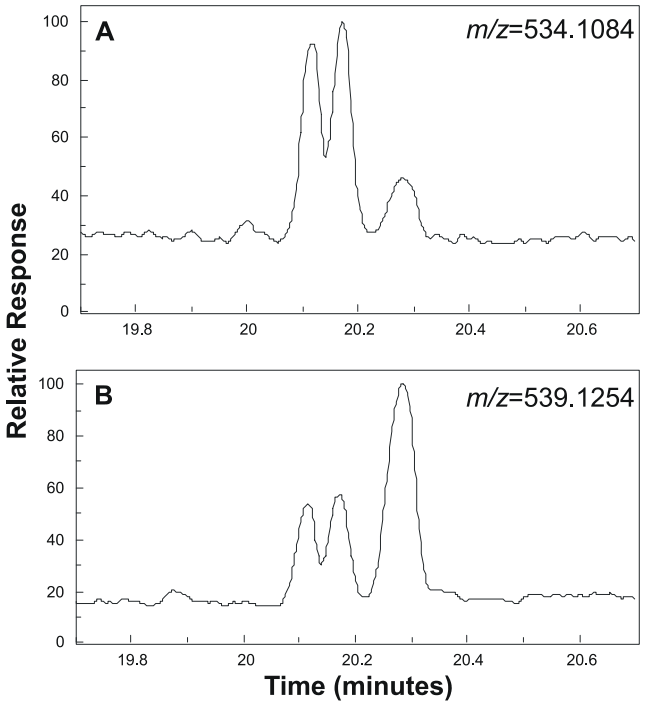

Figure 13. GC-ECNCI-MS chromatograms of (A) THBVal in a globin sample from a Chinese DMF worker (123 mg globin) and of (B) the external standard, THB[$^{13}C_5$]Val-PFPTH. In each chromatogram, the first peak is isomer I, the second peak is isomer II, and the third peak contains isomers III and IV.

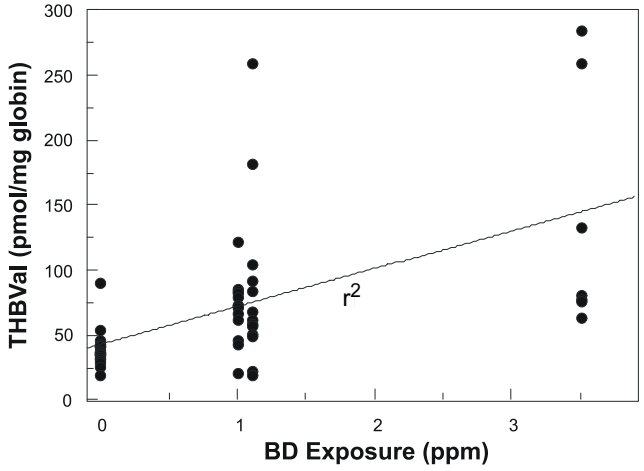

Figure 14. Relation between THBVal levels in Chinese workers and environmental air measurements of BD. The filled circles represent individuals. The nonexposed group (0 ppm BD) includes 25 workers, the polymerization group (1.0 ppm BD) includes 13 workers, the process control group (1.1 ppm BD) includes 13 individuals, and the DMF group (3.5 ppm BD) includes 7 workers.

Hayes and colleagues (1996) found that the median amount of BD in the environment (expressed as 6-hour time-weighted averages) of the polymerization workers was 1.0 ppm, whereas it was 3.5 ppm in the DMF work areas. The process analysts working on routine maintenance had median air concentrations of 1.1 ppm BD, but when pumps and related equipment were being repaired, the BD concentration was as high as 45 ppm. Figure 14 shows individual worker values for total THBVal plotted against these environmental values. The slope of the regression line was significantly different from zero ($p < 0.0001$) and demonstrated greater concentrations of THBVal adducts associated with increased BD exposure ($r^2 = 0.33$).

DISCUSSION

BD is an important industrial chemical used in the manufacture of synthetic rubber and thermoplastic resins and has a potential occupational exposure to over 50,000 American workers. In addition, BD is present in mobile-source emissions, cigarette smoke, and ambient air. In view of the clear carcinogenic potential of BD in laboratory animals, strong evidence of carcinogenic potential in humans during the manufacturing process of

styrene-butadiene rubber, and limited evidence of carcinogenic potential of BD monomer in humans, good measures of exposure must be obtained in order to assess accurately the human risk of cancer from BD exposure. Likewise, better indicators of which metabolites are formed and available to bind to DNA in humans and laboratory animals will promote a better understanding of critical mechanisms of action that need to be used for improved risk assessment.

With this understanding the present research project was undertaken. This laboratory has been one of the leading research groups investigating the DNA adducts formed by BD and the mutagenic consequences of such adduction. At the time this research was proposed, no methods had been developed to quantitate either DNA adducts or hemoglobin adducts of BDO_2. This represented a major gap in our understanding because BDO_2 had been shown to be much more mutagenic than either BDO or BDO-diol (Cochrane and Skopek 1994), yet all measures of BD-related adducts in DNA and hemoglobin had focused on BDO.

During the first year of the project, we applied published methods using a modified Edman degradation of the *N*-terminal valine (Osterman-Golkar et al. 1991, 1993) to the identification and quantitation of HBVal globin. This approach was successful in mice and rats exposed to high concentrations of BD (1,000 ppm, 13 weeks at 6 hours/day, 5 days/week). The long duration of exposure resulted in HBVal adducts that represented steady-state concentrations. $B6C3F_1$ mice had higher concentrations of HBVal adducts than did rats, but male mice (~12,400 pmol/g globin) did not differ significantly from female mice (~16,500 pmol/g globin). We found that male Sprague-Dawley rats had ~5,500 pmol/g globin and females had ~8,700 pmol/g. The higher numbers of adducts in females is consistent with the metabolism studies of Thornton-Manning and colleagues (1997). The lack of statistical significance between males and females may be due to the limited sample sizes. Previous studies of rats and mice have not used the same exposure regimen; however, in all studies mice have had higher numbers of HBVal adducts than rats. Osterman-Golkar and colleagues (1991) reported ~3,000 pmol HBVal adducts/g globin in male Wistar rats exposed to 1,000 ppm BD (2 weeks at 6 hours/day, 5 days/week). The same group reported ~900 pmol HBVal/g globin in male Sprague-Dawley rats exposed to 100 ppm for 4 weeks, and ~3,750 pmol/g in male $B6C3F_1$ mice. Albrecht and colleagues (1993) found ~300 pmol HBVal/g globin after exposure of female Wistar rats to 500 ppm BD for 5 days, and ~500 pmol/g after exposure to 1,300 ppm BD. The same authors reported ~1,700 and ~2,700 pmol/g globin in $CB6F_1$ mice exposed to the same concentrations of BD for 5 days (at 6 hours/day). Albrecht and colleagues (1993) were not able to resolve the two diastereomers of HBVal, whereas all of the other studies were based on combining the data for the two peaks.

In contrast to our success in measuring HBVal in highly exposed rodents, we were not able to detect this adduct in human samples. The problem stemmed from at least two factors. First, the concentration of HBVal in humans was much lower than that present in our BD-exposed rats and mice. Second, background was very high in the chromatograms, even when using high-resolution (10,000 ppm) MS. In the experiment using 100 mg of globin of a pooled sample from four Chinese workers whose exposure groups were unknown to us, we performed multiple HPLC separations on the derivatized product using the same conditions as for standards (see Methods and Study Design). The process took several days, but we succeeded in monitoring HBVal at 2.8 and 1.9 pmol/g globin for isomers I and II (see Figure 11). The resolution of these low concentrations was excellent. However, the method was completely unsuitable for routine analysis of human samples due to the time constraints associated with HPLC clean-up.

The high-resolution mass spectrometer that was used in our experiments was also capable of doing MS/MS analyses, so we attempted to measure one of the human samples by monitoring m/z 374 to 318 in order to reduce the background and to improve our signal-to-noise ratio. Although the background was dramatically reduced, we were unable to measure reliably the number of HBVal adducts. The major reason for this is thought to lie in the particular instrument used, which has a relatively hot source that caused fragmentation of the analyte as it came into the source. Thus, there was minimal m/z 374 available to undergo collision-induced dissociation to form m/z 318. Unfortunately, a number of the samples from Chinese workers were consumed during the HBVal studies and were not available for the THBVal studies. The limited HBVal data we did generate are consistent with HBVal data reported by others: that is, occupational exposures similar to those of the Chinese workers (1 to 3 pmol/g globin) (Osterman-Golkar et al. 1991).

During the last year of this project, we entered into a collaboration with Siv Osterman-Golkar to examine some samples from an independent study for THBVal. Dr. Osterman-Golkar had developed a GC-MS/MS method for THBVal that was later reported by Pérez and colleagues (1997). We made minor modifications to the method and applied high-resolution MS for quantitation. Whereas the GC-MS/MS method could only resolve the THBVal

stereoisomers into two peaks, we were readily able to resolve three peaks. The background in chromatograms was markedly lower than that experienced for HBVal. Furthermore, we found much higher amounts of THBVal in both animal and human samples than we had found for HBVal. In fact, it was possible to demonstrate THBVal in globin from unexposed rats, mice, and humans. Blood samples from a squirrel monkey, dog, calf, and horse were also examined and found to contain similar amounts of THBVal.

The number of THBVal adducts was highest in mice exposed to 1,000 ppm BD for 13 weeks, with females having ~56,000 pmol/g globin and males 79,000 pmol/g. Male rats had ~10,000 pmol/g globin, but female rats had ~22,000 pmol/g. Likewise, we were able to show clear increases in THBVal in female mice exposed to 1,250 ppm BD for 1, 5, or 10 days. There are presently no exposure-response data in rats or mice exposed to lower concentrations of BD. Molecular dosimetry studies on THB-guanine adducts in DNA of mice and rats exposed to 0, 20, 62.5, or 625 ppm BD for 4 weeks have demonstrated strong supralinear exposure-response curves (Koc et al. 1999). In contrast to the THB-guanine exposure-response data, BDO-guanine response was linear from 20 to 625 ppm BD. The number of THB-guanine adducts showed only small increases between 62.5 and 625 ppm BD in rats. In mice, the exposure response suggests biphasic formation, with a sharply increasing response between 0 and 62.5 ppm followed by a less steep slope, but not to the point of saturation of metabolic activation as in the rat. It is likely that a similar exposure response will follow for THBVal. Although the ratio of THBVal:HBVal adducts ranged between 2 and 3 in rats and 4 and 6 in mice exposed to 1,000 ppm BD, this may change markedly at lower exposures. The ratio of THB-guanine:BDO-guanine at 625 ppm was between 5 and 6 in rats and between 12 and 17 in mice, but at lower concentrations it ranged between 15 and 26 in rats and between 27 and 38 in mice.

This project clearly demonstrates the ability of THBVal to serve as a biomarker for human exposure to BD. Exposure-related increases in THBVal were demonstrated in the study of Chinese workers. The number of THBVal adducts was significantly different for all exposed versus unexposed individuals. In addition, the increases showed a good correlation with workplace exposure monitoring at levels ranging from 1 to 3.5 ppm BD.

Perhaps the greatest surprise was the demonstration of THBVal adducts in 70% of the unexposed individuals. The average number of adducts in U.S. volunteers was 36 pmol/g globin for nonsmokers, whereas for Chinese females with no exposure it was 37 pmol/g globin. Considering that 2 of the 13 volunteers and 6 of the 24 Chinese females were below the limit of detection, these averages are actually overestimates. Additional studies with adequate amounts of globin will be needed to depict fully the extent of interindividual variability. Nevertheless, it is highly likely that at least a part of these THBVal adducts arises from endogenous sources. Support in humans for this interpretation also comes from measurements of the MI metabolite in urine of unexposed individuals (Bechtold et al. 1994). This metabolite arises from glutathione conjugation with BD-diol (see Figure 1). Whether the source of BD-diol is from normal physiology or another endogenous chemical that forms THBVal and MI remains unknown. Support from animal studies for an endogenous source of THBVal adducts is provided by the finding of these adducts in six species other than humans. Ambient air concentrations of BD cannot be the source of the THBVal adducts in all of these cases because neither Chinese nor U.S. smokers had significantly different numbers of THBVal adducts. Furthermore, cigarette smoke contains much higher concentrations of BD than does ambient air, which is measured consistently at less than 1 ppb.

Pérez and colleagues (1997) suggest that THBVal arises predominantly from BDO-diol. They arrived at that conclusion in part by the observation that BDO_2 administration gave different THBVal adducts than did BDO-diol. A schematic in their paper, as well as the discussion about this point, were used to support this conclusion. Their schematic was incorrect in one regard, in that it did not show the *meso*-BDO_2. The BDO_2 that they administered consisted only of racemic BDO_2. *meso*-BDO_2 is clearly formed by human microsomes (Krause and Elfarra 1997) and would be expected to form the two stereoisomers that Pérez and colleagues (1997) only associated with BDO-diol exposure. Thus, there is no way to determine which contribution of THBVal comes from BDO-diol and which portion comes from BDO_2. Rydberg and colleagues (1996) have demonstrated the rapid formation of a cyclic adduct with BDO_2 and valinamide in vitro that would be specific for BDO_2. Unfortunately, this adduct cannot be analyzed using a modified Edman reaction and no other ultrasensitive method is presently known. The development of such a method would clearly be desirable because identification of the molecular dose of BDO_2 would greatly improve our understanding of the contribution of specific BD metabolites in carcinogenesis.

As a result of continued methods development, globin samples are now identically processed for the detection of HBVal and THBVal up to the acetylation step for THBVal using Method II. In this way, the same sample can be split for analysis of both analytes from a single derivatization. It

is necessary to employ a GC-MS/MS instrument for the analysis of HBVal. Since the time this research was conducted, we have acquired a new Finnegan TSQ 7000 that performs GC-MS/MS and has a cooler source. The methods developed under this research project are highly suitable for measuring HBVal and THBVal in BD-exposed and unexposed individuals.

In summary, this research project has provided a new, highly sensitive method for measuring THBVal using GC and high-resolution MS. This adduct can be used as a biomarker of BD exposure in an occupational setting (and is being used in the transitional epidemiologic study of Professor Albertini being sponsored by the Health Effects Institute). The research has also pointed out that our current understanding of BD metabolism is inadequate. Past studies have focused on BDO and BDO_2 but have ignored BDO-diol. The data from this project and similar studies being conducted in our laboratory using DNA from tissues of rats and mice exposed to lower concentrations of BD strongly suggest that BDO-diol is the major electrophile produced in rats, mice, and humans. Using literature values for BD metabolites and the DNA adduct data, we were able to calculate that ~95% of the THB-guanine adducts arose from BDO-diol (Koc et al. 1999). This is also likely to be the case for THBVal adducts. Accurate assessments of cancer risk in humans from exposure to BD cannot be made until a better understanding of the metabolism of BD is gained in laboratory animals and humans. Molecular dosimetry studies on DNA and hemoglobin adducts of BD will provide critical data for developing this improved understanding.

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REFERENCES

Adler I-D, Cao J, Filser JG, Gassner P, Kessler W, Kliesch U, Neuhauser-Klaus A, Nusse M. 1994. Mutagenicity of 1,3-butadiene inhalation in somatic and germinal cells of mice. Mutat Res 309:307–314.

Adler I-D, Filser JG, Gassner P, Kessler W, Schoneich J, Schriever-Schwemmer G. 1995. Heritable translocations induced by inhalation exposure of male mice to 1,3-butadiene. Mutat Res 347:121–127.

Adler I-D, Filser J, Gonda H, Schriever-Schwemmer G. 1998. Dose response study of 1,3-butadiene-induced dominant lethal mutations and heritable translocations in germ cells of male mice. Mutat Res 381:85–92.

Albrecht OE, Filser JG, Neumann H-D. 1993. Biological monitoring of 1,3-butadiene: Species differences in haemoglobin binding in rat and mouse. In: Butadiene and Styrene: Assessment of Health Hazards (Sorsa M, Peltonen K, Vanino H, Hemminki K, eds.) pp. 135–142. International Agency for Research on Cancer, Lyon, France.

Autio K, Renzi L, Catalan J, Albrecht OE, Sorsa M. 1994. Induction of micronuclei in peripheral blood and bone marrow erythrocytes of rats and mice exposed to 1,3-butadiene by inhalation. Mutat Res 309:315–320.

Bechtold WE, Strunk MR, Chang I-Y, Ward JB Jr, Henderson RF. 1994. Species differences in urinary butadiene metabolites: Comparisons of metabolite ratios between mice, rats, and humans. Toxicol Appl Pharmacol 127:44–49.

Bergmark E. 1997. Hemoglobin adducts of acrylamide and acrylonitrile in laboratory workers, smokers and nonsmokers. Chem Res Toxicol 10:78–84.

Boogaard PJ, Bond JA. 1996. The role of hydrolysis in detoxification of 1,2:3,4-diepoxybutane by human, rat, and mouse liver and lung in vitro. Toxicol Appl Pharmacol 141:617–627.

Boogaard PJ, Summer SCJ, Bond JA. 1996. Glutathione conjugation of 1,2:3,4-diepoxybutane in human liver and rat and mouse liver and lung in vitro. Toxicol Appl Pharmacol 136:307–316.

Brinkworth MH, Anderson D, Hughes JA, Jackson JI, Yu T-W, Nieschlag E. 1998. Genetic effects of 1,3-butadiene on the mouse testis. Mutat Res 397:67–75.

Citti L, Gervasi PG, Turchi G, Bellucci G, Bianchini R. 1984. The reaction of 3,4-epoxy-1-butene with deoxyguanosine and DNA in vitro: Synthesis and characterization of the main adducts. Carcinogenesis 5:47–52.

Cochrane JE, Skopek TR. 1994. Mutagenicity of butadiene and its epoxide metabolites: I. Mutagenic potential of 1,2-epoxybutene, 1,2,3,4-diepoxybutane, and 3,4-epoxy-1,2-butanediol in cultured human lymphoblasts. Carcinogenesis 15:713–717.

Delzell E, Sathiakumar N, Hovinga M, Macaluso M, Julian J, Larson R, Cole P, Muir DC. 1996. A follow-up study of synthetic rubber workers. Toxicology 113:182–189.

Divine BJ, Hartman CM. 1996. Mortality update of butadiene production workers. Toxicology 113:169–181.

Hayes RB, Xi L, Bechtold WE, Rothman N, Yao M, Henderson R, Zhang L, Smith MT, Zhang D, Wiemels J, Dosemeci M, Yin S, O'Neill JP. 1996. *hprt* mutation frequency among workers exposed to 1,3-butadiene in China. Toxicology 113:100–105.

Himmelstein MW, Acquavella JF, Recio L, Medinsky MA, Bond JA. 1997. Toxicology and epidemiology of 1,3-butadiene. Crit Rev Toxicol 27:1–108.

International Agency for Research on Cancer. 1987. IARC Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs, Vol. 1 to 42 (Supplement 7), 1,3-Butadiene (Group 2B). International Agency for Research on Cancer, Lyon, France.

Koc H, Tretyakova NY, Walker VE, Henderson RF, Swenberg JA. 1999. Molecular dosimetry of N-7 guanine adduct formation in mice and rats exposed to 1,3-butadiene. Chem Res Toxicol 12:566–574.

Koivisto P, Adler I-D, Pacchierotti F, Peltonen K. 1998. DNA adducts in mouse testis and lung after inhalation exposure to 1,3-butadiene. Mutat Res 397:3–10.

Koivisto P, Adler I-D, Sorsa M, Peltonen K. 1996. Inhalation exposure of rats and mice to 1,3-butadiene induces N^6-adenine adducts of epoxybutene detected by ^{32}P-postlabeling and HPLC. Environ Health Perspect 104(Suppl 3):655–657.

Krause RJ, Elfarra AA. 1997. Oxidation of butadiene monoxide to *meso*- and (±)-diepoxybutane by cDNA-expressed human cytochrome P450s and by mouse, rat, and human liver microsomes: Evidence for preferential hydration of *meso*-diepoxybutane in rat and human liver microsomes. Arch Biochem Biophys 337:176–184.

La DK, Swenberg JA. 1997. Carcinogenic alkylating agents. In: Comprehensive Toxicology, Vol. 12, Chemical Carcinogens and Anticarcinogens (Sipes IG, McQueen CA, Gandolfi AJ, eds.) pp. 111–140. Elsevier Science, Oxford, England.

Legator MS, Au WW, Ammenhauser MM, Ward JB Jr. 1993. Elevated somatic cell mutant frequencies and altered DNA repair responses in nonsmoking workers exposed to 1,3-butadiene. In: Butadiene and Styrene: Assessment of Health Hazards (Sorsa M, Peltonen K, Vainio H, Hemminki K, eds.) pp. 253–263. International Agency for Research on Cancer, Lyon, France.

Leuratti C, Jones NJ, Marafante E, Kostiainen R, Peltonen K, Waters R. 1994. DNA damage induced by the environmental carcinogen butadiene: Identification of a diepoxybutane-adenine adduct and its detection by ^{32}P-postlabeling. Carcinogenesis 15:1903–1910.

Meng Q, Recio L, Wong B, Walker VE. 1996. Comparison of the mutagenic potency of butadiene at HPRT of T-cells following inhalation exposures of B6C3F$_1$ mice and F344 rats. Environ Mol Mutagen 27:48.

Morrow NL. 1990. The industrial production and use of 1,3-butadiene. Environ Health Perspect 86:7–8.

Mowrer J, Törnqvist M, Jensen S, Ehrenberg L. 1986. Modified Edman degradation applied to hemoglobin for monitoring occupational exposure to alkylating agents. Toxicol Environ Chem 11:215–257.

National Institute for Occupational Safety and Health. 1990. National Occupational Exposure Survey (1981–1983). National Institute for Occupational Safety and Health, Cincinnati, OH.

National Toxicology Program, U.S. Public Health Service, U.S. Department of Health and Human Services. 1984. Toxicology and Carcinogenesis Studies of 1,3-Butadiene (CAS No. 106-99-0) in B6C3F$_1$ Mice (Inhalation Studies). NTP TR 288, NIH Pub. No. 84-2544. National Toxicology Program, Research Triangle Park, NC.

National Toxicology Program, U.S. Public Health Service, U.S. Department of Health and Human Services. 1993. Technology Report on the Toxicology and Carcinogenesis Studies of 1,3-Butadiene. NTP TR 434, NIH Pub. No. 93-3165. National Toxicology Program, Research Triangle Park, NC.

Neagu I, Koivisto P, Neagu C, Kostiainen R, Stenby K, Peltonen K. 1995. Butadiene monoxide and deoxyguanosine alkylation products at the N7-position. Carcinogenesis 16:1809–1813.

Norppa H, Hirvonen A, Järventaus H, Uusküla M, Tasa G, Ojajarvi A, Sorsa M. 1995. Role of GSTT1 and GSTMI genotypes in determining individual sensitivity to sister chromatid exchange induction by diepoxybutane in cultured human lymphocytes. Carcinogenesis 16:1261–1264.

Osterman-Golkar S, Bond JA, Ward JB Jr, Legator MS. 1993. Use of hemoglobin adducts for biomonitoring exposure to 1,3-butadiene. In: Butadiene and Styrene: Assessment of Health Hazards (Sorsa M, Peltonen K, Vanino H, Hemminki K, eds.) pp.127–134. International Agency for Research on Cancer, Lyon, France.

Osterman-Golkar S, Kautiainen A, Bergmark E, Hakansson K, Maki-Paakkanen J. 1991. Hemoglobin adducts and urinary mercapturic acids in rats as biological indicators of butadiene exposure. Chem Biol Interact 80:291–302.

Owen PE, Glaister JR, Gaunt IF, Pullinger DH. 1987. Inhalation toxicity studies with 1,3-butadiene: 3. Two-year toxicity/carcinogenicity study in rats. Am Ind Hyg Assoc J 48:407–413.

Pérez HL, Lahdetie J, Landin HH, Kilpelainen I, Koivisto P, Peltonen K, Osterman-Golkar S. 1997. Haemoglobin adducts of epoxybutanediol from exposure to 1,3-butadiene or butadiene epoxides. Chem Biol Interact 105:181–198.

Ranasinghe A, Scheller N, Wu K-Y, Upton PB, Swenberg JA. 1998. Application of gas chromatography/electron capture negative chemical ionization high-resolution mass spectrometry for analysis of DNA and protein adducts. Chem Res Toxicol 11:520–526.

Rydberg P, Magnusson A-L, Zorcec V, Granath F, Törnqvist M. 1996. Adducts to *N*-terminal valines in hemoglobin from butadiene metabolites. Chem Biol Interact 101:193–205.

Sabourin PJ, Burka LT, Bechtold WE, Dahl AR, Hoover MD, Chang IY, Henderson RF. 1992. Species differences in urinary butadiene metabolites: Identification of 1,2-dihydroxy-4-(*N*-acetylcysteinyl)butane, a novel metabolite of butadiene. Carcinogenesis 13:1633–1638.

Sangaiah R, Christova-Gueorguieva NI, Ranasinghe A, Tretyakova N-Y, Gold A, Ball LM, Swenberg JA. 1997. Synthesis of perdeuterated analogues of the epoxide metabolites of butadiene: 1,2-Epoxybut-3-ene-d_6 and 1,2,3,4-diepoxybutane-d_6. J Label Compd Radiopharm 39:731–739.

Seaton MJ, Follansbee MH, Bond JA. 1995. Oxidation of 1,2-epoxy-3-butene to 1,2:3,4-diepoxybutane by cDNA-expressed human cytochromes P450 2E1 and 3A4 and human, mouse and rat liver microsomes. Carcinogenesis 16:2287–2293.

Stephanou G, Russo A, Vlastos D, Andrianopoulos C, Demopoulos NA. 1997. Micronucleus induction in somatic cells of mice as evaluated after 1,3-butadiene inhalation. Mutat Res 381:11–20.

Tates AD, van Dam FJ, de Zwart FA, Darroudi F, Natarajan AT, Rossner P, Peterkova K, Peltonen K, Demopoulos NA, Stephanou G, Vlachodimitriopoulos D, Šrám R. 1996. Biological effect monitoring in industrial workers from the Czech Republic exposed to low levels of butadiene. Toxicology 111:91–99.

Tates AD, van Dam FJ, de Zwart FA, van Teylingen CMM, Natarajan AT. 1994. Development of a cloning assay with high cloning efficiency to detect induction of 6-thioguanine-resistant lymphocytes in spleen of adult mice following in vivo inhalation exposure to 1,3-butadiene. Mutat Res 309:299–306.

Tates AD, van Dam FJ, van Teylingen CM, de Zwart FA, Zwinderman AH. 1998. Comparison of induction of HPRT mutations by 1,3-butadiene and its metabolites 1,2-epoxybutene and 1,2,3,4-diepoxybutane in lymphocytes from spleen of adult male mice and rats in vivo. Mutat Res 397:21–36.

Thornton-Manning JR, Dahl AR, Bechtold WE, Griffith WC Jr, Henderson RF. 1997. Comparison of the disposition of butadiene epoxides in Sprague-Dawley rats and B6C3F_1 mice following a single and repeated exposures to 1,3-butadiene via inhalation. Toxicology 123:125–134.

Törnqvist M. 1994. Epoxide adducts to *N*-terminal valine of hemoglobin. Methods Enzymol 231:650–657.

Törnqvist M, Mowrer J, Jensen S, Ehrenberg L. 1986. Monitoring of environmental cancer initiators through hemoglobin adducts by a modified Edman degradation method. Anal Biochem 154:255–266.

Tretyakova N, Lin Y, Sangaiah R, Upton PB, Swenberg JA. 1997a. Identification and quantitation of DNA adducts from calf thymus DNA exposed to 3,4-epoxy-1-butene. Carcinogenesis 18:137–147.

Tretyakova N-Y, Lin Y-P, Upton PB, Sangaiah R, Swenberg JA. 1996. Macromolecular adducts of butadiene. Toxicology 113:70–76.

Tretyakova N, Sangaiah R, Yen T-Y, Gold A, Swenberg JA. 1997b. Adenine adducts with diepoxybutane: Isolation and analysis in exposed calf thymus DNA. Chem Res Toxicol 10:1171–1179.

Tretyakova NY, Sangaiah R, Yen T-Y, Swenberg JA. 1997c. Synthesis, characterization, and in vitro quantitation of N-7-guanine adducts of diepoxybutane. Chem Res Toxicol 10:779–785.

Tretyakova NY, Walker VE, Swenberg JA. 1998. Formation and persistence of DNA adducts in liver of rats and mice exposed to 1,3-butadiene. Toxicologist 42:180.

U.S. Environmental Protection Agency. 1994. Locating and Estimating Air Emissions from Sources of 1,3-Butadiene (September 1994 draft). Office of Air Quality Planning and Standards, Research Triangle Park, NC.

Uusküla M, Järventaus H, Hirvonen A, Sorsa M, Norppa H. 1995. Influence of GSTMI genotype on sister chromatid exchange induction of styrene-7,8-oxide and 1,2-epoxy-3-butene in cultured human lymphocytes. Carcinogenesis 16:947–950.

Walker VE, Fennel TR, Upton PB, MacNeela JP, Skopek TR, Shuker DEG, Swenberg JA. 1993. Molecular dosimetry of ethylene oxide: Formation and persistence of 7-(2-hydroxyethyl)guanine in DNA following repeated exposures of rats and mice. Cancer Res 52:4328–4334.

Ward JB Jr, Ammenhauser MM, Bechtold WE, Whorton EB Jr, Legator MS. 1994. *hprt* mutant lymphocyte frequencies in workers at a 1,3-butadiene production plant. Environ Health Perspect 102:79–85.

Wiencke JK, Pemble S, Ketterer B, Kelsey KT. 1995. Gene deletion of glutathione *S*-transferase: Correlation with induced genetic damage and potential role in endogenous mutagenesis. Cancer Epidemiol Biomarkers Prev 4:253–259.

Xiao Y, de Stoppelaar JM, Hoebee G, Schriever-Schwemmer G, Adler I-D, Tates AD. 1996. Analysis of micronuclei induced by 1,3-butadiene and its metabolites using fluorescence in situ hybridization. Mutat Res 354:49–57.

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Dr. Hayes became a tenured senior investigator in the Division of Cancer Epidemiology and Genetics at the National Cancer Institute in 1992. His work involves occupational cohorts of subjects that provide unique opportunities to study cancer risks associated with high-level exposure to chemicals, such as benzene, 1,3-butadiene, and formaldehyde, that are also found in the general environment but at substantially lower levels. He uses cohort and case-control studies in the general population to investigate population risks for cancer and to identify gene-environment interactions.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Hayes RB, Zhang L, Yin S, Swenberg JA, Wiencke J, Bechtold WE, Yao M, Rothman N, Haas R, O'Neill PO, Wiemels J, Dosemeci M, Li G, Smith M. 1999. Genotoxic markers among butadiene-polymer workers in China. Carcinogenesis 21:55–62.

ABBREVIATIONS AND OTHER TERMS

amu	atomic mass unit
ANOVA	analysis of variance
BD-diol	1,2-dihydroxy-3-butene
BD	1,3-butadiene
BDO	1,2-epoxy-3-butene
BDO_2	1,2,3,4-diepoxybutane
BDO-diol	1,2-dihydroxy-3,4-epoxybutane
CYP2E1	cytochrome P450 2E1
DDW	distilled deionized water
DMF	dimethyl formamide
ECNCI	electron-capture negative chemical ionization
GC-MS	gas chromatography–mass spectrometry
GST	glutathione *S*–transferase
HBVal	*N*-(2-hydroxy-3-butenyl)valine
HEVal	*N*-(2-hydroxyethyl)valine
HPLC	high-pressure liquid chromatography
$KHCO_3$	potassium bicarbonate
LC-ESI-MS	liquid chromatography–negative ion electrospray–mass spectrometry
M	mass [in figures only]
MI	1,2-dihydroxy-4-(*N*-acetylcysteinyl)butane
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
Na_2CO_3	sodium carbonate
NaOH	sodium hydroxide
PFPITC	pentafluorophenylisothiocyanate
PFPTH	pentafluorothiohydantoin
r^2	regression coefficient
RBCs	red blood cells
SIM	selected ion monitoring
THBVal	*N*-(2,3,4-trihydroxybutyl)valine

COMMENTARY ON PARTS IV AND V
Health Review Committee

HEI

INTRODUCTION

Rodent bioassays clearly demonstrate that 1,3-butadiene (BD)* is carcinogenic at multiple sites in male and female mice and rats and that mice are significantly more sensitive than rats (Owen et al. 1987; National Toxicology Program 1984, 1993; IARC 1999). Studies in mouse and rat tissues suggest that species differences in metabolic pathways and in the rates at which BD is activated and detoxified could explain why mice are more sensitive than rats to the carcinogenic effects of BD (Osterman-Golkar et al. 1991; Himmelstein et al. 1994, 1997; Richardson et al. 1999). Some researchers have suggested that humans may be more similar to rats than to mice in the way they metabolize BD (Bond et al. 1995); if this is true, it will have important implications for risk assessment. The studies conducted by Drs. Blair and Swenberg, as part of HEI's butadiene research program, were funded to develop a better understanding of the biological mechanisms leading to BD carcinogenicity (Blair) and, in particular, to develop methods to measure biological markers that could assess BD exposure (Blair, Swenberg). Such biomarkers would not only have the potential to be applied as measures of internal dose in epidemiologic studies, but would also serve as a measure of BD's bioactivation and its reaction with macromolecules.

During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Reports and in the Health Review Committee's Commentary. The following Commentary is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the studies, by pointing out alternative explanations for data, and by placing the Investigators' Reports into scientific and regulatory perspective.

SCIENTIFIC BACKGROUND

Biomarkers are used by researchers as indicators of past or current exposures to chemicals, including air pollutants. Biomarkers can be measured in breath, blood, urine, or tissue. Depending on the type of biomarker, it can indicate a level of exposure to a carcinogen or other air pollutant, the dose received in the body or at a critical target site, a molecular event caused by the chemical or its metabolites, a biological effect defined as a health endpoint of interest, or a susceptibility to disease. Biomarkers can also function as normalizing elements among species or among levels of exposure when comparing dose-response relations. For example, a relevant biomarker along the causal pathway of cancer could be compared in rats and mice exposed to different concentrations of a chemical. If this biomarker is also found and validated in occupationally exposed individuals, it may help us understand the relation between exposure level and cancer risk in people.

Drs. Ian Blair and James Swenberg were funded under the Health Effects Institute's air toxics research program to develop and validate methods for measuring butadiene biomarkers. (See the Preface to this Research Report for background information on BD and a description of HEI's research program in this area.) The biomarkers that these investigators examined in their independent studies were DNA adducts and hemoglobin adducts, respectively.

Adducts are molecules bound covalently to macromolecules such as DNA or proteins. DNA adducts are formed spontaneously between a reactive molecule and a DNA base. Most cells contain enzymes that recognize lesions in DNA, such as DNA adducts, and repair the damage. These enzymes excise the base with the adduct and replace it with a normal base. The excised adduct can then be excreted in the urine. Adducts that are not repaired are referred to as persistent adducts. The rate at which the adducts are excised and the DNA is repaired are important factors when assessing DNA adduct levels in a tissue at a given point in time. Reactive molecules can also spontaneously form adducts with proteins. Protein adducts differ from DNA adducts in that they do not cause mutations and are not typically removed by a repair mechanism, but persist for the lifetime of the protein.

Both DNA and protein adducts have the potential to be used as biomarkers of exposure to chemical pollutants. However, before such biomarkers can be used in epidemiologic studies, sensitive, specific, and reliable measurement techniques need to be developed and validated. The Blair and Swenberg reports describe the development of analytical techniques for detecting and quantifying adducts derived from the active metabolites of BD and the initial validation of these techniques using samples from animals (Blair and Swenberg) and humans (Swenberg) exposed to BD.

* A list of abbreviations appears at the end of each Investigators' Report.

TECHNICAL EVALUATION OF BLAIR'S REPORT

In response to RFA 93-1, Dr. Ian Blair (then of Vanderbilt University and now of the University of Pennsylvania) submitted an application entitled "Molecular Dosimetry of Conjugated Dienes and Aliphatic Aldehydes".

The original objectives of Blair's proposed study were to determine whether:

1. a sensitive method, using liquid chromatography and tandem mass spectrometry (LC–MS/MS), could be developed to quantify DNA adducts formed after exposure to mobile-source emissions. The major focus would be directed toward quantifying, in urine and target organs of laboratory animals, the DNA and crotonaldehdye adducts formed after exposure to BD or to racemic mixtures of 1,2-epoxy-3-butene (BDO) or 1,2,3,4-diepoxybutane (BDO_2). (A racemic mixture is a compound made up of equal amounts of isomers with opposite configurations at one or more asymmetric centers; all references to BDO or BDO_2 hereafter indicate the racemic forms.)
2. DNA adducts could be identified in liver and other tissues of rats and mice exposed to BD and its metabolites; and
3. the persistence of DNA adducts could be monitored by measuring elimination of adducts in the animals' urine.

The HEI Health Research Committee noted that the investigator was well qualified, had proposed to use state-of-the-art techniques, and had addressed some important objectives of the RFA. Although Blair's original study design did not include analysis of human samples, the Research Committee thought that developmental work was necessary to identify the adducts in animals before proceeding to human samples. The investigators completed the most important study objectives, the development of methods for characterizing BD-derived DNA adducts in tissues and urine. However, information on crotonaldehyde-derived adducts and adducts in tissues other than liver was not included in the final report.*

STUDY DESIGN AND METHODS

Blair's report describes the development of LC–MS/MS methods for analyzing N7-guanine adducts in the tissue and urine of animals exposed to BD. The study design is straightforward and the methods are described in detail in the Investigators' Report. The investigators successfully applied modern chromatographic and mass spectrometric procedures to quantify adducts of the reactive metabolites of BD, in particular those arising from BDO and BDO_2. The chemistry of the adducts was studied and the adducts quantified in several systems: calf-thymus DNA exposed to BDO or BDO_2; DNA from human TK6 cells exposed to BDO in culture; DNA from liver tissues from mice and rats exposed to BD by inhalation; and urine from BD-exposed rodents. The work was conducted in an analytically rigorous manner with appropriate internal controls and validation processes.

Methods Development

Blair and colleagues synthesized authentic N7-guanine adducts of the BD metabolites BDO and BDO_2; these adducts were N7-2-hydroxy-3-butenyl-1-guanine (2HB1G) and N7-1-hydroxy-3-butenyl-2-guanine (1HB2G) for BDO, and N7-(2,3,4-trihydroxybutyl)guanine (THBG) for BDO_2. They also synthesized isotope-labeled N7-guanine adducts of BDO and BDO_2 to use as internal standards. Then they developed LC–MS/MS methods to analyze BDO-guanine and BDO_2-guanine adducts in DNA. Using calf-thymus or TK6 cells (a human lymphoblastoid cell line), they performed preliminary quantitative studies using their methods to identify the amount of each metabolite incorporated into DNA in vitro and the stability of the respective adducts. This was done by incubating calf-thymus DNA with BDO or BDO_2 for up to 12 hours and TK6-cell DNA with BDO for 24 hours and assessing the persistence of adducts over time.

The LC–MS/MS protocol used for the analyses in this study was a three-step process. First, the molecule of interest, for example a DNA adduct, was purified by LC. The eluted adduct was injected into a mass spectrometer (the first MS stage) using electron spray ionization; this procedure added a charge to the molecule, but did not fragment it. In the second MS stage, selected reaction monitoring, the molecule of interest was fragmented by collision-induced dissociation and the fragments were separated. These fragmentation products from the unknown molecule are compared with those from a purified known molecule (the external standard), which has undergone the same process, to identify the molecule of interest.

Methods Validation

Incubation of calf-thymus DNA with BDO for up to 24 hours resulted in the formation of equal amounts of the two DNA adducts 2HB1G and 1HB2G. Incubation of

* Dr. Blair's three-year study, "Molecular Dosimetry of Conjugated Dienes and Aliphatic Aldehydes," began in October 1994 with total expenditures of $618,938. The Investigators' Report from Dr. Blair and his colleagues was received for review in July 1998. A revised report, received in December 1998, was accepted for publication in February 1999.

calf-thymus DNA with BDO_2 resulted in the formation of the adduct THBG. Blair found (1) no regioselectivity (a property of a reaction in which a single structural isomer is favored) in the formation of any adduct after calf-thymus DNA was incubated with BDO; and (2) the rates of spontaneous depurination (nonenzymatic removal) of any of the three DNA adducts examined in this study were similar.

Blair validated his methods by adding known amounts of 2HB1G and 1HB2G, and of their stable isotope-labeled analogs (as internal standards), to samples of DNA that had not been exposed to BDO and analyzing these samples by the same DNA-isolation process used for the experimental samples. Adducts formed with calf-thymus or TK6-cell DNA and with the internal and external standards were analyzed by LC–MS/MS. The adducts that had been released from the DNA by spontaneous depurination as well as urinary BD adducts were similarly analyzed.

Application of Methods for Analyzing Adducts from Laboratory Animals

In the next phase of the study, Blair applied his newly developed techniques to: (1) measure BD-derived adducts in urine (adducts derived from spontaneous depurination and DNA repair); (2) assess BD adducts in liver (persistent adducts); and (3) determine the biological half-lives of adducts in urine and liver. The investigator used tissue and urine samples from F344 rats and $B6C3F_1$ mice that had been exposed by inhalation to 1,250 ppm BD for 6 hours/day, 5 days/week for up to 2 weeks.* They collected urine samples every 24 hours from rats and mice for 3 days after exposure. Rats and mice were killed at 1, 5, or 10 days of exposure, or 1, 3, or 6 days after exposure.

The purpose of this part of the study was to determine whether the liver and urine concentrations of 2HB1G, 1HB2G, and THBG in rats and mice would show differences that are consistent with the known difference between rats and mice in their carcinogenic response to BD exposure (i.e., mice are more sensitive than rats to carcinogenesis after exposure to BD). Because 2HB1G and 1HB2G are formed from the reaction of BDO with guanine moieties in DNA, and (±)-THBG and *meso*-THBG are formed from reactions of either BDO_2 or 1,2-dihydroxy-3,4-epoxybutane (BDO-diol) with guanine, the amounts of the different adducts in liver and urine could provide a measure of the relative formation of BD metabolites in vivo. Knowing which metabolites are formed in what relative quantities could help explain the species difference in sensitivity to BD exposure. Furthermore, if it could be established that the amounts of specific adducts formed indicate the identity and quantity of specific BD metabolites formed in vivo, such a correlation could be used to develop an assay for a urinary biomarker of BD exposure that would provide information on both the internal dose and the biologically effective dose.

* The animal exposures for Dr. Blair's study were carried out at the Chemical Industry Institute of Toxicology in collaboration with Drs. Leslie Recio and Vernon Walker (New York State Department of Health).

RESULTS AND INTERPRETATION

Blair and colleagues demonstrated that the LC–MS/MS method could be used successfully to detect and analyze DNA adducts derived from BD metabolites in the liver and urine of mice and rats exposed to high concentrations of BD (1,250 ppm for 6 hours/day, 5 days/week for 2 weeks). Because relatively little of the body's guanine pool exists as free guanine and because RNA is repaired at a slower rate than DNA, it is reasonable to assume that urinary BD-guanine adducts come primarily from excised and spontaneously depurinated DNA. However, the proportion of adducts derived from target organs such as lungs relative to nontarget organs such as liver cannot be assessed.

Blair measured the appearance and clearance of four adducts formed between BD metabolites and guanine: 2HB1G, 1HB2G, (±)-THBG, and *meso*-THBG. The results obtained for mouse and rat liver and urine are summarized in Table 1.

The two N7-guanine adducts derived from BDO, 2HB1G and 1HB2G, are a regioisomeric pair (meaning they differ in the carbon position that forms a bond with the N7 position of guanine; see the Preface to this Research Report for more information on stereoisomers) (Citti et al. 1984).

Table 1. Levels of N7-Guanine Adducts in Mice and Rats Exposed to 1,250 ppm BD as Measured in the Blair Study

N7-Guanine Adduct	Source	Liver[a] (adducts/10^6 bases)		Urine[b] (ng adduct/24-hour urine sample)	
		Mice	Rats	Mice	Rats
2HB1G	BDO	1	0.25	5	10
1HB2G	BDO	4	2	110	220
(±)-THBG	(±)-BDO_2 or BDO-diol	4	1.5	30	60
meso-THBG	*meso*-BDO_2 or BDO-diol	2	0.75	ND[c]	ND

[a] Peak values at 10 days after exposure.

[b] Peak values at 2 days after exposure.

[c] ND = Not determined.

From his studies of liver tissue, Blair reported that the levels of 2HB1G and 1HB2G were higher in mouse-liver DNA than in rat-liver DNA during the 2-week exposure period and for 6 weeks after exposure. Regioselective differences for these persistent adducts were observed in both species; 8 times more 1HB2G than 2HB1G was found in rat-liver DNA and 4 times more 1HB2G than 2HB1G in mouse-liver DNA. In both species, 1HB2G adducts from the liver declined rapidly after exposure ended. Blair's studies of adducts in urine demonstrated the opposite relation between species: The levels of 2HB1G and 1HB2G excreted in urine were twofold higher in rats than mice. Nevertheless, the same regioselectivity was observed in both species in that 20 times more 1HB2G than 2HB1G was excreted. However, no difference between species was noted in the relative amounts of regioisomers of urinary metabolites resulting from depurination and excision repair of BDO-derived adducts.

The N7-guanine adduct derived from BDO_2 or BDO-diol, THBG, was found in two diastereomeric forms: (±)-THBG and *meso*-THBG. On the basis of this research, it was not possible to distinguish whether the diastereomers were derived from BDO_2 or from BDO-diol. (±)-THBG can be formed by a reaction between DNA and either BDO_2 or BDO-diol; *meso*-THBG can be formed by the reaction between DNA and either *meso*-BDO_2, which is one of the stereoisomers of BDO_2 formed by oxidation of BDO, or BDO-diol.

Blair's studies of liver tissue showed that the concentrations of both THBG adducts (the number of adducts per 10^6 DNA bases) detected in mouse-liver DNA were more than two times higher than the concentrations detected in rat-liver DNA, although *meso*-THBG appeared in lesser amounts than (±)-THBG. In both species, (±)-THBG peaked after 10 days of BD exposure, declined by about 50% 1 day after exposure ended, and declined much more slowly after that. In contrast, the data suggest that the biological half-life of *meso*-THBG in mouse- and rat-liver DNA may be longer than that of (±)-THBG. Of potential biological interest is the observation that *meso*-THBG appeared to decline more slowly in mouse-liver than rat-liver DNA.

The studies of urine revealed that in both species, the levels of (±)-THBG peaked 2 days after exposure ended and declined the next day. The amounts excreted in the 24-hour urine samples were twofold higher in rat urine than in mouse urine during the time course of the experiment. The presence or absence of *meso*-THBG in urine was not determined.

DISCUSSION

The analytical methods developed by Blair and colleagues for characterizing DNA adducts could contribute substantially toward elucidating which BD metabolites are responsible for DNA damage and ultimately carcinogenesis. Furthermore, the ability to identify and characterize regioisomers offers investigators a powerful analytical method to assess the isomers' participation in the sequence of events that results in carcinogenesis.

One example of the usefulness of the methods developed by Blair and coworkers was the detection of *meso*-THBG in mouse- and rat-liver DNA from animals exposed to BD. The amount of *meso*-THBG detected in rat-liver DNA was much less than that in mouse-liver DNA, but its persistence suggests a very long biological half-life, especially compared with that of (±)-THBG in liver. The biological relevance of *meso*-THBG formation in rat- and mouse-liver DNA requires confirmation and further study, but Blair's results suggest that this adduct may be important for assessing the carcinogenic potential of BD.

In addition, the observation of regioselectivity in the formation of BDO-DNA adducts is interesting and warrants further studies to determine the biological significance of this selectivity. The relative amounts of 2HB1G, 1HB2G, and THBG adducts in urine may reflect preferential synthesis or preferential degradation of BDO or BDO_2 adducts, or some combination of these processes.

One puzzling result was the seeming disappearance of the 2HB1G adduct and the persistence of the 1HB2G adduct in liver tissue from mice and rats exposed to BD. Blair's in vitro studies showed that 2HB1G and 1HB2G were formed from BDO at approximately equal rates in both calf-thymus and TK6-cell DNA. Both regioisomers spontaneously depurinated at equal rates in calf-thymus DNA; whereas in TK6-cell DNA, 2HB1G disappeared more rapidly, possibly as a result of both spontaneous depurination and excision-repair. Although it is difficult to evaluate the persistence of adducts in vitro because of the large standard deviation of the data, it appears that both adducts are formed and nonenzymatically degraded at equal rates. In vivo, however, for both BD-exposed rats and mice, more 1HB2G was found in liver DNA and more 1HB2G was excreted in urine. The investigators tried to reconcile the inconsistencies between the in vitro and in vivo data by suggesting that the 2HB1G in liver DNA is rapidly repaired, and therefore one would not expect to see it as a stable adduct. However, they also presented data that showed the half-lives of the two adducts in rat- and mouse-liver DNA are almost the same. If it is true that 2HB1G is more quickly repaired than 1HB2G in liver and is therefore found in lower concentrations, it would

follow that more 2HB1G adduct (having been excised in the liver) would appear in the urine, where in fact there is less. The authors recognize this discrepancy and suggest that the 2HB1G adduct was somehow metabolized to a form that was not detected by their analytical methods.

One limitation in interpreting Blair's data on the differences between DNA adducts in urine is the lack of information on tissue origin of the adducts. The investigators had originally proposed to examine several tissues, but measured only liver adducts. Therefore, it was not possible to determine how different tissues may have contributed to the adducts found in the urine, nor whether repair and spontaneous depurination rates were consistent from tissue to tissue. Furthermore, whereas measuring the BD metabolite adducts in tissue samples as purines (i.e., guanine) is appropriate, measuring only purines in urine samples may limit interpretation of the data because adducts in urine come from other sources in addition to DNA.

These experiments were conducted in animals exposed to a relatively high concentration of BD (1,250 ppm). Although the investigators conducted dose-response experiments that incorporated lower doses of BD, those tissues and urine samples were not analyzed. More work needs to be done to evaluate the sensitivity of the assay Blair and his colleagues developed, and to apply it to ambient environmental exposures (0.5 to 10 ppb) and the low range of occupational concentrations (less than 10 to 370 ppm) of BD (Melnick and Kohn 1995).

TECHNICAL EVALUATION OF SWENBERG'S REPORT

For the BD research program, the HEI Health Research Committee originally funded four studies (Henderson, Recio, Walker, and Blair) from the applications received in response to RFA 93-1. As the program developed, Dr. Walker of the New York State Department of Health, who was studying the mutagenic potencies and mutational spectra of BD and its metabolites, requested additional funds to collaborate with Dr. James Swenberg of the University of North Carolina at Chapel Hill. Dr. Swenberg was developing mass spectrometric methods to measure hemoglobin adducts derived from BD metabolites and proposed analyzing these adducts in tissues from rodents and humans exposed to BD. The Research Committee approved limited funds and requested a separate application from Dr. Swenberg before recommending full funding. Dr. Swenberg's application ("Hemoglobin Adducts as Biomarkers of Butadiene Exposure and Metabolism") was peer-reviewed, discussed by the Committee, and funded as the fifth study in the butadiene program.*

The specific aims of Swenberg's study were to determine whether:

1. new mass spectrometric (MS) methods could be developed and validated to analyze adducts formed between either of two BD metabolites (BDO or BDO_2) and hemoglobin;
2. these methods would be sufficiently sensitive to detect adduct formation in rats and mice exposed to multiple doses of BD, BDO, or BDO_2; and
3. hemoglobin adduct levels measured in samples from BD-exposed workers would correlate with their exposure history.

Although not one of the original aims, BDO-diol was found to have a significant role in hemoglobin adduct formation and therefore was studied as well.

Overall, Swenberg and colleagues achieved their aim of developing high-resolution MS methods to quantify hemoglobin adducts that result from reactive intermediates of BD. However, not all the experiments that were originally proposed were carried out. Because of the unexpected unavailability of some samples, the investigators were able to examine red blood cell (RBC) samples only from animals exposed to two high doses of BD. Therefore, the investigators could not provide dose-response data. The investigators did not provide information on adducts formed in animals exposed to BDO or BDO_2. Instead, they moved directly to examining human tissues.

STUDY DESIGN AND METHODS

The overall objective of this research project was to develop a high-resolution MS method to quantify hemoglobin adducts derived from the known reactive metabolites of BD (BDO, BDO-diol, and BDO_2) with the *N*-terminal valines of hemoglobin.

For the animal studies, Swenberg used blood samples from rats and mice. Crl:CD®BR rats and $B6C3F_1$/CrlBR mice were exposed to 1,000 ppm BD by inhalation (6 hours/day, 5 days/week, for 13 weeks) at the Haskell Laboratory of E. I. DuPont de Nemours and Company. $B6C3F_1$ mice were exposed to 1,250 ppm BD (6 hours/day, 5 days/week, for 1, 5, or 10 days) at the Chemical Industry Institute of Toxicology as part of experiments designed by two other HEI-funded investigators (Walker and Henderson). The levels of adducts were presumed to be at

* Dr. Swenberg's two-year study began in April 1996 with total expenditures of $137,911. The Investigators' Report from Dr. Swenberg and his colleagues was received for review in July 1998. A revised report, received in January 1999, was accepted for publication in February 1999.

steady state because the 13-week exposure duration exceeded the lifespan of the RBC. For the human studies, Swenberg and colleagues obtained blood samples from a study of Chinese workers in the polybutadiene industry, conducted by Dr. Richard Hayes of the National Cancer Institute, and from volunteers at the University of North Carolina.

The investigators isolated globin from RBC hemoglobin using a slight modification of standard procedures (Mowrer et al. 1986; Walker et al. 1993). The RBCs were lysed and globin was isolated and purified. To remove the *N*-terminal valine, the globin was subjected to a modified Edman degradation procedure (Törnqvist et al. 1986). Because this procedure had been well studied and documented, many of the difficulties had already been resolved, which allowed Swenberg's initial period of methods development to proceed to analyzing tissue samples in a relatively short time.

When this study was initiated, it was known that the major products of the reaction of BDO with the amino group of the *N*-terminal valine of globin were two diastereomers (isomers I and II) of *N*-(2-hydroxy-3-butenyl)valine (HBVal) (Osterman-Golkar 1991, 1993). The reaction between BDO_2 or BDO-diol and globin, however, had not been previously studied and no method had been developed to quantify the reaction product. Swenberg used four stereoisomers (isomers I–IV) of *N*-(2,3,4-trihydroxybutyl)valine (THBVal) as markers. Isomers I–IV are most likely the products of valine reacting with the 1-position of BDO-diol.

For the HBVal assays, Swenberg prepared an authentic internal standard of globin alkylated with [$^2H_6$]BDO; for the THBVal assays, he used a synthesized external standard, THB[$^{13}C_5$]Val. Using internal standards permitted accurate quantification of the detected adducts. The investigators treated the *N*-terminal valine adducts with pentafluorophenylisothiocyanate (PFPITC) or pentafluorothiohydantoin (PFPTH) to increase the sensitivity of the MS detection methods. The rodent hemoglobin adducts were then analyzed by gas chromatography–mass spectrometry (GC-MS) with no further treatment; the human blood samples were purified on C_{18} columns and acetylated before GC-MS. LC-MS was used only to analyze the synthesized standards, and GC-MS was used to analyze the experimental samples, internal and external standards as controls, and calibration standards.

RESULTS AND INTERPRETATION

Swenberg and colleagues successfully developed new methods to analyze hemoglobin adducts derived from BD metabolites in blood samples from animals and humans exposed to BD. The method for measuring THBVal represents an important advance because no procedure had previously been developed for measuring adducts from BDO_2 or BDO-diol, both of which may be important metabolites in BD carcinogenisis.

The amounts of BD-derived adducts were higher in mouse globin than in rat globin: HBVal was approximately two times as high (14,480 vs 7,085 pmol/g globin) and THBVal was about four times as high (67,500 vs. 15,625 pmol/g globin). The amounts of HBVal Swenberg found attached to globin were consistent with other published values (Osterman-Golkar et al. 1991). Swenberg's data also demonstrated that THBVal formation in female mice exposed to 1,250 ppm BD increased linearly over a 10-day exposure period.

To test the sensitivity of their methods and their applicability for assessing human exposure to BD, Swenberg and colleagues analyzed 87 samples of blood taken from Chinese workers in a polybutadiene manufacturing plant. These samples included both BD-exposed and unexposed individuals. Additional samples from 13 unexposed volunteers at the University of North Carolina were also analyzed. Both the Chinese and American samples included specimens from smokers and nonsmokers (BD is found in cigarette smoke). The mean total THBVal detected in blood samples from unexposed U.S. subjects was 36.3 pmol/g globin in nonsmokers and 40.2 pmol/g globin in smokers, and from control Chinese workers was 39.3 pmol/g globin. These values were compared with a mean total of 88.3 pmol/g globin in blood samples from the BD-exposed Chinese workers, a statistically significant difference. Further breakdown of the data suggested a dose-response relation in the Chinese worker group. Overall, the results demonstrate that methods have been developed for monitoring hemoglobin adducts derived from exposure to BD.

The investigators found that it was possible to measure low levels of HBVal in a sample of blood pooled from four Chinese workers, but not in a single sample. They suggest the reason for the low sensitivity in human blood samples is that the electronspray source, which operates at a very high temperature, fragments the adduct and it is thus lost for analysis. (Other investigators have not had this problem using a different instrument [Ostermann-Golkar et al. 1991, 1993].) The limit of detection for HBVal was about 1 pmol/g globin with Swenberg's method and instrumentation. The mouse and rat studies Swenberg conducted to validate the assay had been performed with animals exposed to high concentrations of BD (1,000 or 1,250 ppm), and thus had measured HBVal amounts in the range of thousands of picomoles per gram of globin. These levels are much higher than those predicted to form after

human exposure to the lower ambient or occupational concentrations of BD.

In contrast to the results with HBVal, Swenberg was able to detect THBVal in blood samples from single workers (rather than needing to pool samples from several workers). On the basis of THBVal levels, therefore, he could differentiate between Chinese workers classified as unexposed or exposed to BD. Because actual data on BD exposure levels were not presented in the Investigators' Report, no conclusion could be drawn about the linearity of adduct formation and exposure. However, this research has certainly provided methods for measuring THBVal adducts in human blood samples after exposure to BD at expected occupational levels.

DISCUSSION

Swenberg and colleagues successfully developed MS methods for measuring hemoglobin adducts derived from BD metabolites, in particular those arising from BDO-diol or BDO_2. This is important because, as the investigators note, BDO_2 and BDO-diol are major metabolites of BD in mice, a species sensitive to tumor induction after exposure to BD. Moreover, BDO_2 is more mutagenic than BDO, and BDO-diol is believed to be formed at levels higher than BDO. At the time this study was funded, methods had been established for measuring hemoglobin adducts arising from BDO, the initial BD oxidation product (Osterman-Golkar et al. 1991, 1993), but not from BDO_2 or BDO-diol. Thus, Swenberg's assay and the methods being concurrently developed by other investigators (Osterman-Golkar et al. 1996, 1998; Pérez et al. 1997) provide researchers with the necessary tools to conduct a more complete assessment of the risk posed by exposure to BD, taking into account adducts that can be formed by all three metabolites (BDO, BDO_2, and BDO-diol).

Swenberg and his colleagues improved the sensitivity of the THBVal assay to the stage where increases in BD-derived adducts over background levels can be measured. However, as Swenberg points out, the background level of THBVal adducts was high in subjects with no occupational exposure. Whether this represents an exposure to BD in the ambient environment, endogenous formation, or an artifact is unknown and is worthy of further investigation. The median value of THBVal for control subjects was about 36 pmol/g globin, at least half of the median value for occupationally exposed individuals, and in several individuals higher than the value for exposed subjects. Thus, the reasons for the high background level will need to be explored before THBVal data can be used as a biomarker for exposure to BD. With the methods recently developed, more extensive studies can be conducted with both animals and humans in which exposures can be related to the extent and type of hemoglobin adduct formed.

SUMMARY

In two parallel studies, Blair and Swenberg developed new methods for quantifying biomarkers of exposure to BD.

The Blair Investigators' Report describes: (1) the in vitro synthesis of adducts formed between BD metabolites and guanine as standards for analysis of DNA adducts generated in vivo; (2) validation of the identity and the amount of each adduct formed; (3) generation of adducts in isolated DNA and in DNA from cultured cells; (4) identification and quantification, via liver tissues and urine, of the adducts generated in vivo in mice and rats exposed to 1,250 ppm BD; and (5) demonstration that the methods are capable of distinguishing regioisomers. The results of this study suggest that mice form more DNA adducts after exposure to BD than rats do, whereas rats may repair BD-derived DNA adducts faster than mice. The method developed to quantify depurinated N7-guanine adducts of BD in urine could provide a noninvasive means to monitor genotoxic metabolism of BD in exposed human populations. In addition, it can be used to assess the importance of BD stereoisomers in the sequence of molecular events leading to cancer. However, to achieve these goals the sensitivity of the assay needs to be enhanced considerably.

The Swenberg Investigators' Report describes: (1) in vivo synthesis of adducts formed between BD metabolites and the *N*-terminal valine of hemoglobin; (2) cleavage of these adducts from globin by the Edman degradation procedure; (3) validation of the identity and amounts of these adducts; (4) formation and measurement of hemoglobin adducts in mice and rats exposed to BD; and (5) preliminary application of these methods to human samples. The investigators' only setback of note was the inability to measure HBVal derived from BDO in human samples. Their major contribution lies in having generated a method to measure THBVal formed by BDO_2 or BDO-diol that is applicable to human blood samples. The investigators' pilot study successfully demonstrated that their method was sufficiently sensitive to detect hemoglobin adducts in people exposed to occupational levels of BD. The next step will be to demonstrate an exposure-response relation over a range of exposure doses in animals and in humans.

The methods developed by Blair and Swenberg may prove to be powerful tools for assessing human exposure to BD. The adducts found in urine represent BD metabolites that have attached to DNA, which makes it possible to link carcinogen exposure with DNA damage. On the

basis of the data obtained by Blair and colleagues, the processes of BD activation and DNA adduct formation and repair occur rapidly; thus, BD-derived DNA adducts reflect recent exposures and the time between exposure and measurement will be critical in interpreting results. Because hemoglobin adducts have a considerably longer biological half-life than most DNA adducts or urinary metabolites, the levels of these protein adducts can reflect accumulation of exposure over several months. Thus, simultaneous measurement of DNA- and hemoglobin-adducts offers an opportunity to assess the impact of short-term exposure and long-term cumulative exposure (over a period of months) on the carcinogenic potential of BD in humans. The success of this strategy will depend, however, on greatly improving the sensitivity of detecting DNA adducts in urine. In contrast, assays for hemoglobin adducts developed by Swenberg and others appear to be sufficiently sensitive for human studies.

Both methods were developed using tissues from animals exposed to high doses of BD over relatively long periods of time. Overall, higher adduct levels were observed in tissues and fluids from mice than in those from rats. On the basis of the findings from these studies, it would now be useful to characterize the dose-response relations at low-level exposure levels in mice and rats to ascertain the differences in adduct production at high and low exposure levels both within and across species. Such information would also be applicable as a guide for assessing low-level exposure in humans. In addition, it would be helpful to determine if adduct formation and excretion in mice and rats is affected by such variables as low-level continuous exposure or intermittent short-term high-level exposure. Finally, on the basis of the preliminary studies conducted by Swenberg and associates in which they measured BD adducts in unexposed humans, it seems to be critical that sufficient data be collected from control populations to establish baseline values from which investigators can assess the impact of low-level exposures to BD on adduct formation.

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REFERENCES

Bond JA, Recio L, Andjelkovich D. 1995. Epidemiological and mechanistic data suggest that 1,3-butadiene will not be carcinogenic to humans at exposures likely to be encountered in the environment or workplace. Carcinogenesis 16:165–171.

Himmelstein MW, Turner MJ, Asgharian B, Bond JA. 1994. Comparison of blood concentrations of 1,3-butadiene and butadiene epoxides in mice and rats exposed to 1,3-butadiene by inhalation. Carcinogenesis 1(5):1479–1486.

Himmelstein MW, Acquavella JF, Recio L, Medinsky MA, Bond JA. 1997. Toxicology and epidemiology of 1,3 butadiene. Crit Rev Toxicol 27:1–108.

International Agency for Research on Cancer. 1999. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Re-Evaluation of Some Organic Chemicals, Hydrazine and Hydrogen Peroxide, Volume 71, Part I, pp. 109-225. IARC, Lyon, France.

Melnick RL, Kohn MC. 1995. Mechanistic data indicate that 1,3-butadiene is a human carcinogen. Carcinogenesis 16:157–163.

National Toxicology Program. 1984. Technology Report on the Toxicology and Carcinogenesis Studies of 1,3-Butadiene in Mice (Inhalation Studies). NTP TR 288, NIH Publication 84-2544. National Toxicology Program, Research Triangle Park, NC.

National Toxicology Program. 1993. Technology Report on the Toxicology and Carcinogenesis Studies of 1,3-Butadiene. NTP TR 434, NIH Publication 93-3165. National Toxicology Program, Research Triangle Park, NC.

Osterman-Golkar S, Bond JA, Ward JB Jr, Legator MS. 1993. Use of hemoglobin adducts for biomonitoring exposure to 1,3-butadiene. In: Butadiene and Styrene: Assessment of Health Hazards (Sorsa M, Peltonen K, Vanino H, Hemminki K, eds.) pp. 127–134. IARC Scientific Publications Number 127. International Agency for Research on Cancer, Lyon, France.

Osterman-Golkar S, Kautiainen A, Bergmark E, Hakansson K, Maki-Paakkanen J. 1991. Hemoglobin adducts and urinary mercapturic acids in rats as biological indicators of butadiene exposure. Chem Biol Interact 80:291–302.

Owen PE, Glaister JR, Gaunt IF, Pullinger DH. 1987. Inhalation toxicity studies of 1,3-butadiene: 3. Two year toxicity/carcinogenicity study in rats. Am Ind Hyg Assoc J 48:407–413.

Pérez HL, Lahdetie J, Landin HH, Kilpelainen I, Koivisto P, Peltonen K, Osterman-Golkar S. 1997. Haemoglobin adducts of epoxybutanediol from exposure to 1,3-butadiene or butadiene epoxides. Chem Biol Interact 105:181–198.

Richardson KA, Peters MM, Wong BA, Megens RH, van Elburg PA, Booth ED, Boogaard PJ, Bond JA, Medinsky MA, Watson WP, van Sittert NJ. 1999. Quantitative and qualitative differences in the metabolism of ^{14}C-1,3-butadiene in rats and mice: Relevance to cancer susceptibility. Toxicol Sci 49:186–201.

Törnqvist M, Mowrer J, Jensen S, Ehrenberg L. 1986. Monitoring of environmental cancer initiators through hemoglobin adducts by a modified Edman degradation method. Anal Biochem 154:255–266.

U.S. Environmental Protection Agency. 1998. Health Risk Assessment of 1,3-Butadiene: External Review Draft. EPA/600/P-98/001A. Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC.

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