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Genotoxicity of 1,3-Butadiene and Its Epoxy Intermediates

Vernon E. Walker, Dale M. Walker, Quanxin Meng, Jacob D. McDonald, Bobby R. Scott, Steven K. Seilkop, David J. Claffey, Patricia B. Upton, Mark W. Powley, James A. Swenberg, and Rogene F. Henderson

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with a Critique by the HEI Health Review Committee

Research Report 144

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ABOUT HEI

The Health Effects Institute is a nonprofit corporation chartered in 1980 as an independent research organization to provide high-quality, impartial, and relevant science on the effects of air pollution on health. To accomplish its mission, the institute

- Identifies the highest-priority areas for health effects research;
- Competitively funds and oversees research projects;
- Provides intensive independent review of HEI-supported studies and related research;
- Integrates HEI's research results with those of other institutions into broader evaluations; and
- Communicates the results of HEI's research and analyses to public and private decision makers.

HEI receives half of its core funds from the U.S. Environmental Protection Agency and half from the worldwide motor vehicle industry. Frequently, other public and private organizations in the United States and around the world also support major projects or certain research programs. HEI has funded more than 280 research projects in North America, Europe, Asia, and Latin America, the results of which have informed decisions regarding carbon monoxide, air toxics, nitrogen oxides, diesel exhaust, ozone, particulate matter, and other pollutants. These results have appeared in the peer-reviewed literature and in more than 200 comprehensive reports published by HEI.

HEI's independent Board of Directors consists of leaders in science and policy who are committed to fostering the public–private partnership that is central to the organization. The Health Research Committee solicits input from HEI sponsors and other stakeholders and works with scientific staff to develop a Five-Year Strategic Plan, select research projects for funding, and oversee their conduct. The Health Review Committee, which has no role in selecting or overseeing studies, works with staff to evaluate and interpret the results of funded studies and related research.

All project results and accompanying comments by the Health Review Committee are widely disseminated through HEI's Web site (*www.healtheffects.org*), printed reports, newsletters, and other publications, annual conferences, and presentations to legislative bodies and public agencies.

ABOUT THIS REPORT

Research Report 144, *Genotoxicity of 1,3-Butadiene and Its Epoxy Intermediates,* presents a research project funded by the Health Effects Institute and conducted by Dr. Vernon E. Walker of the Lovelace Respiratory Research Institute, Albuquerque, New Mexico, and his colleagues. This report contains three main sections.

The HEI Statement, prepared by staff at HEI, is a brief, nontechnical summary of the study and its findings; it also briefly describes the Health Review Committee's comments on the study.

The Investigators' Report, prepared by Walker et al., describes the scientific background, aims, methods, results, and conclusions of the study.

The Critique is prepared by members of the Health Review Committee with the assistance of HEI staff; it places the study in a broader scientific context, points out its strengths and limitations, and discusses remaining uncertainties and implications of the study's findings for public health and future research.

This report has gone through HEI's rigorous review process. When an HEI-funded study is completed, the investigators submit a draft final report presenting the background and results of the study. This draft report is first examined by outside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the Health Review Committee, an independent panel of distinguished scientists who have no involvement in selecting or overseeing HEI studies. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, as necessary, to revise their report. The Critique reflects the information provided in the final version of the report.

HEI STATEMENT

Synopsis of Research Report 144

Genotoxicity of 1,3-Butadiene and Its Metabolites

BACKGROUND

1,3-Butadiene (BD) is used extensively in the chemical industry (e.g., for synthetic rubber production) and is also part of motor vehicle exhaust and cigarette smoke. BD is listed by the U.S. Environmental Protection Agency (EPA) as a mobile-source air toxic and was recently reclassified by EPA as a human carcinogen via inhalation exposure. This classification was based on occupational exposures of workers in the butadiene rubber industry who showed higher levels of certain leukemias compared with the general population. In 2002, the EPA cited additional evidence for carcinogenicity from chronic inhalation studies with rodents, but the carcinogenic and mutagenic potency of BD is different in mice and rats, which complicates extrapolating results from rodents to humans for risk assessment purposes. Therefore, toxicologic research in the past decade has focused on how the metabolism of BD differs between mice and rats (and how human metabolism compares) and on assessing the mutagenicity of reactive epoxide metabolites of BD in these two species as well as in human cells in vitro. The major metabolites of interest have been the monoepoxide (BDO), the diepoxide (BDO_2), and the epoxydiol (BDO-diol).

Dr. Walker proposed to investigate several unresolved issues surrounding BD metabolites, such as (1) the role of stereoisomerism (differences in the three-dimensional shape of chemical compounds) in the mutagenicity of the metabolites, (2) how much the nonreactive intermediary metabolite BD-diol contributes to the formation of reactive metabolites, and (3) the mutagenic potency of BD in mice and rats exposed to high and low levels of BD. The study would focus on differences in mutagenic potency of BD between rodent species, age groups, and sexes. The HEI Health Research Committee recommended funding the study because it would address many of the outstanding questions regarding the mutagenicity of BD and its metabolites and that information would be important in extrapolating results from animal studies to humans.

APPROACH

The study comprised a series of experiments in which male and female F344 rats and B6C3F1 mice, 4 to 5 weeks or 8 to 9 weeks old, were exposed via inhalation to BD or its metabolites BDO_2 and BD-diol. Exposures lasted for 6 hours/day, 5 days/week, for 2 to 4 weeks; control groups were exposed to filtered air. Upon completion of the exposures, lymphocytes (T cells) were collected from the spleen to determine mutations in the *Hprt* gene. (Rare cells with mutations in the *Hprt* gene can be selected from normal cells by adding 6-thioguanine to the culture medium. It kills normal cells because they incorporate it into their DNA, but does not affect the mutant cells, which are unable to incorporate it.)

First, *Hprt* mutant frequencies were determined in rodents exposed to 3 or 1250 ppm BD to assess sex and age differences in mutagenic response to BD at low and high concentrations. These data were compared with data obtained from earlier studies by Dr. Walker.

Second, rodents were exposed to 2 or 4 ppm BDO_2 of the *meso* form (which does not have optical properties) and killed at different intervals to assess the time course of the formation of *Hprt* mutant cells and to evaluate differences between sexes and species. These data were compared with data from rats exposed to a mixture of + and – forms of BDO_2 (which differ in how they refract light) in earlier studies by Dr. Walker, thereby assessing the role of stereoisomerism of BDO_2 in the mutagenicity of BD.

Third, rodents were exposed to BD-diol (6, 18, or 36 ppm) to assess the extent and time course of *Hprt* mutant frequencies. (Because BD-diol is converted to BDO-diol, exposure to BD-diol allowed that metabolic pathway to be investigated without

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr. Vernon Walker at Lovelace Respiratory Research Institute, Albuquerque, NM, and colleagues. Research Report 144 contains both the detailed Investigators' Report and a Critique on the study prepared by the Institute's Health Review Committee.

interference by BDO_2, which is also converted to BDO-diol.) Additional data were obtained on levels of BD-diol and other metabolites after exposure to BD (200 ppm) or BD-diol (24 or 36 ppm). The investigators developed a sensitive method to analyze BD-diol levels in plasma specifically for this purpose.

Fourth, cells derived from male rodents exposed to BD (1250 ppm), *meso*-BDO_2 (2 or 4 ppm), or BD-diol (6, 18, or 36 ppm) were cloned and propagated for molecular analyses of mutation spectra to assess whether the kinds of mutations (such as point mutations or larger deletions) differ among BD and its metabolites.

RESULTS AND INTERPRETATION

This study has provided important data on the mutagenic potency of BD at low exposure concentrations (3 ppm). In addition, Dr. Walker and colleagues confirmed and extended earlier observations that (1) female rodents are more susceptible than male rodents to BD exposure, (2) mice are more susceptible than rats to inhaled BD, and (3) rodents 4 to 5 weeks old are more sensitive to inhaled BD than animals 8 to 9 weeks old. The investigators attributed this age-related effect to differences in thymus activity and movement of T cells through the body. Dr. Walker and colleagues showed that in both species the contribution of the metabolites BD-diol and BDO-diol to mutagenicity induced by BD exposure is most prominent at high BD concentrations, whereas the metabolite BDO_2 is probably more important at lower concentrations. They found that stereochemistry did not play a role in mutagenicity induced by BDO_2; however, the in vivo role of the stereochemistry of the monoepoxide metabolites (BDO and BDO-diol) remains to be established.

Future studies should include a comprehensive analysis of metabolite concentrations, kinetics, and adduct levels, coupled with in vivo analysis of mutant frequencies. In addition to knowing each metabolite's mutagenic potency, the process of formation and elimination of metabolites in and from the body should be carefully assessed, as well as the extent to which metabolites form DNA adducts that may cause mutations or other genetic damage. In the past, several studies have investigated these issues, but mostly in isolation. A more comprehensive approach could provide the information needed to determine which metabolites are critical to the toxicity of BD and how best to extrapolate results from rodents to humans, especially at low levels of exposure (3 ppm and below).

INVESTIGATORS' REPORT

Genotoxicity of 1,3-Butadiene and Its Epoxy Intermediates

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ABSTRACT

Current risk assessments of 1,3-butadiene (BD*) are complicated by limited evidence of its carcinogenicity in humans. Hence, there is a critical need to identify early events and factors that account for the heightened sensitivity of mice to BD-induced carcinogenesis and to determine which animal model, mouse or rat, is the more useful surrogate of potency for predicting health effects in BD-exposed humans. HEI sponsored an earlier investigation of mutagenic responses in mice and rats exposed to BD, or to the racemic mixture of 1,2-epoxy-3-butene (BDO) or of 1,2,3,4-diepoxybutane (BDO_2; Walker and Meng 2000). In that study, our research team demonstrated (1) that the frequency of mutations in the hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) gene of splenic T cells from BD-exposed mice and rats could be correlated with the species-related differences in cancer susceptibility; (2) that mutagenic-potency and mutagenic-specificity data from mice and rats exposed to BD or its individual epoxy intermediates could provide useful information about the BD metabolites responsible for mutations in each species; and (3) that our novel approach to measuring the mutagenic potency of a given chemical exposure as the change in *Hprt* mutant frequencies (M*f*s) over time was valuable for estimating species-specific differences in mutagenic responses to BD exposure and for predicting the effect of BD metabolites in each species.

To gain additional mode-of-action information that can be used to inform studies of human responses to BD exposure, experiments in the current investigation tested a new set of five hypotheses about species-specific patterns in the mutagenic effects in rodents of exposure to BD and BD metabolites:

1. Repeated BD exposures at low levels that approach the occupational exposure limit for BD workers (set by the U.S. Occupational Safety and Health Administration) are mutagenic in female mice.
2. The differences in mutagenic responses of the *Hprt* gene to BD in similarly exposed rodents of a given species (reported in various earlier studies) are primarily associated with age-related thymus activity and trafficking of T cells and with sex-related differences in BD metabolism.
3. The mutagenic potency of the stereochemical forms of BD's epoxy intermediates plays a significant role in the species-related mutagenicity of BD.
4. The hydrolysis-detoxification pathway of BD through 1,2-dihydroxy-3-butene (BD-diol) is a major contributor to mutagenicity at high-level BD exposures in mice and rats.
5. Significant and informative species-specific differences in mutation spectra can be identified by examining both large- and small-scale genetic alterations in the *Hprt* gene of BD-exposed mice and rats.

The first four hypotheses were tested by exposing mice and rats to BD, *meso*-BDO_2, or BD-diol and measuring *Hprt*

This Investigators' Report is one part of Health Effects Institute Research Report 144, which also includes a Critique by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Vernon E. Walker, Genetic Toxicology Laboratory, Department of Pathology, 665 Spear Street, Burlington, Vermont 05405.

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

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

M*f*s as the primary biomarker. For this, we used the T-cell–cloning assay of lymphocytes isolated from the spleens of exposed and control (sham-exposed) mice and rats.

The first hypothesis was tested by exposing female B6C3F1 mice (4 to 5 weeks of age) by inhalation for 2 weeks (6 hours/day, 5 days/week) to 0 or 3 ppm BD. *Hprt* M*f*s were measured at the time of peak mutagenic response after exposure for this age of mice. We then compared the resulting data to those from mutagenicity studies with mice of the same age that had been exposed in a similar protocol to higher levels of BD (Walker and Meng 2000). In mice exposed to 3 ppm BD (n = 27), there was a significant 1.6-fold increase over the mean background *Hprt* M*f* in control animals (n = 24, P = 0.004). Calculating the efficiency of *Hprt* mutant induction, by dividing induced *Hprt* M*f*s by the respective BD exposure levels, demonstrated that the mutagenic potency of 3 ppm BD was twice that of 20 ppm BD and almost 20 times that of 625 or 1250 ppm BD in exposed female mice. Sample-size calculations based on the *Hprt* M*f* data from this experiment demonstrated the feasibility of conducting a future experiment to find out whether induced M*f*s at even lower exposure levels (between 0.1 and 1.0 ppm BD) fit the supralinear exposure–response curve found with exposures between 3.0 and 62.5 ppm BD, or whether they deviate from the curve as M*f* values approach the background levels found in control animals.

The second hypothesis was tested by estimating mutagenic potency for female mice exposed by inhalation for 2 weeks to 0 or 1250 ppm BD at 8 weeks of age and comparing this estimate to that reported for female mice exposed to BD in a similar protocol at 4 to 5 weeks of age (Walker and Meng 2000). For these two age groups, the shapes of the mutant splenic T-cell manifestation curves were different, but the mutagenic burden was statistically the same. These results support our contention that the disparity in responses reported in earlier *Hprt*-mutation studies of BD-exposed rodents is related more to age-related T-cell kinetics than to age-specific differences in the metabolism of BD.

The third hypothesis was tested by estimating mutagenic potency for female mice and rats (4 to 5 weeks of age) exposed by inhalation to 2 or 4 ppm *meso*-BDO_2 and comparing these estimates to those previously obtained for female mice and rats of the same age and exposed in a similar protocol to (±)-BDO_2 (Meng et al. 1999b; Walker and Meng 2000). These exposures to stereospecific forms of BDO_2 caused equivalent mutagenic effects in each species. This suggests that the small differences in the mutagenic potency of the individual stereoisomers of BDO_2 appear to be of less consequence in characterizing the sources of BD-induced mutagenicity than the much larger differences between the mutagenic potencies of BDO_2 and the other two BD epoxides (BDO and 1,2-dihydroxy-3,4-epoxybutane [BDO-diol]).

The fourth hypothesis was tested in several experiments. First, female and male mice and rats (4 to 5 weeks of age) were exposed by nose only for 6 hours to 0, 62.5, 200, 625, or 1250 ppm BD or to 0, 6, 18, 24, or 36 ppm BD-diol primarily to establish BD and BD-diol exposure levels that would yield similar plasma concentrations of BD-diol. Second, animals were exposed in inhalation chambers for 4 weeks to 0, 6, 18, or 36 ppm BD-diol to determine the mutagenic potency estimates for these exposure levels and to compare these estimates with those reported for BD-exposed female mice and rats (Walker and Meng 2000) in which similar blood levels of BD-diol had been achieved.

Measurements of plasma concentrations of BD-diol (via a gas chromatography and mass spectrometry [GC/MS] method developed for this purpose) showed these results: First, BD-diol accumulated in a sublinear manner during a single 6-hour exposure to more than 200 ppm BD. Second, BD-diol accumulated in a linear manner during single (6-hour) or repeated (4-week) exposure to 6 or 18 ppm BD and in a sublinear manner with increasing levels of BD-diol exposure. Third, exposure of female mice and rats to 18 ppm BD-diol produced plasma concentrations equivalent to those produced by exposure to 200 ppm BD (exposure to 36 ppm BD-diol produced plasma concentrations of about 25% of those produced by exposure to 625 ppm BD).

In general, 4-week exposure to 18 or 36 ppm BD-diol was significantly mutagenic in female and male mice and rats. The differences in mutagenic responses between the species and sexes were not remarkable, except that the mutagenic effects were greatest in female mice. The substantial differences in the exposure-related accumulation of BD-diol in plasma after rodents were exposed to more than 200 ppm BD compared with the relatively small differences in the mutagenic responses to direct exposures to 6, 18, or 36 ppm BD-diol in female mice provided evidence that the contribution of BD-diol–derived metabolites to the overall mutagenicity of BD has a narrow range of effect that is confined to relatively high-level BD exposures in mice and rats. This conclusion was supported by the results of parallel analyses of adducts in mice and rats concurrently exposed to BD-diol (Powley et al. 2005b), which showed that the exposure–response curves for the formation of *N*-(2,3,4-trihydroxybutyl)valine (THB-Val) in hemoglobin, formation of N7-(2,3,4-trihydroxybutyl)guanine (THB-Gua) in DNA, and induction of *Hprt* mutations in exposed rodents were remarkably similar in shape (i.e., supralinear). Combined, these data suggest that trihydroxybutyl (THB) adducts are good quantitative indicators of BD-induced mutagenicity

and that BD-diol–derived BDO-diol (the major source of the adducts) might be largely responsible for mutagenicity in rodents exposed to BD-diol or to high levels of BD.

The mutagenic-potency studies of *meso*-BDO_2 and BD-diol reported here, combined with our earlier studies of BD, (±)-BDO, and (±)-BDO_2 (Walker and Meng 2000), revealed important trends in species-specific mutagenic responses that distinguish the relative degree to which the epoxy intermediates contribute to mutation induction in rodents at selected levels of BD exposures. These data as a whole suggest that, in mice, BDO_2 largely causes mutations at exposures less than 62.5 ppm BD and that BD-diol–derived metabolites add to these mutagenic effects at higher BD exposures. In rats, it appears that the BD-diol pathway might account for nearly all the mutagenicity at the high-level BD exposures where significant increases in *Hprt* M*f*s are found and cancers are induced. Additional exposure–response studies of hemoglobin and DNA adducts specific to BDO_2, BDO-diol, and other reactive intermediates are needed to determine more definitively the relative contribution of each metabolite to the DNA alkylation and mutation patterns induced by BD exposure in mice and rats.

For the fifth hypothesis, a multiplex polymerase chain reaction (PCR) procedure for the analysis of genomic DNA mutations in the *Hprt* gene of mice was developed. This procedure was used with other established methods for the coincident identification of both large- and small-scale genetic alterations in *Hprt* of expanded mutant T cells isolated from BD-exposed and control rodents.

We combined mutation-spectra data from reverse transcription–polymerase chain reaction (RT–PCR) of complementary DNA (cDNA) and from multiplex PCR of genomic DNA from the *Hprt* mutants; then for each type of mutation identified, we performed statistical analyses of mutant fractions. This combination showed that BD exposure of mice significantly increased the frequencies of (1) nearly all types of base substitutions, (2) single-base deletions and insertions, and (3) all subcategories of deletions. The significantly increased frequency of G•C → C•G and A•T → T•A transversions in *Hprt* of BD-exposed mice was consistent with the occurrence of these same substitutions found to be the predominant *ras* gene mutations in a variety of tumor types that had increased in frequency in earlier carcinogenicity studies of BD in mice. BD exposure of rats produced significant increases in (1) base substitutions only at A•T, (2) single-base insertions, (3) complex mutations, and (4) deletions (mainly 5′ partial and complete gene deletions). Additional molecular analyses of *Hprt* mutants from rodents exposed directly to BDO_2 or BD-diol are needed to determine the source(s) of G•C → C•G and A•T → T•A transversions in both species and to bolster evidence for the species-specific role of these metabolites in causing mutations related to carcinogenesis in rodents exposed to BD.

INTRODUCTION

BD is an indirect alkylating agent with the potential to induce DNA damage, mutation, and cancer (Himmelstein et al. 1997). Long-term animal studies have shown that BD has substantially greater cancer potency in mice than in rats (Huff et al. 1985; Owen et al. 1987; Melnick et al. 1990; Melnick and Huff 1992). However, epidemiologic studies evaluating the effects of BD exposure in humans have not been conclusive (Landrigan 1993; Delzell et al. 1996, 2001; Divine and Hartman 1996). Thus, the question of the relative sensitivity of humans to the hazardous effects of BD is the subject of considerable debate, with some scientists suggesting that the mouse is the appropriate model for human cancer risk assessment (Melnick and Kohn 1995) and others arguing that the rat is more appropriate (Bond et al. 1995). Since the risk assessment of BD exposure is currently complicated by limited evidence of its carcinogenicity in humans and by uncertainties as to which rodent species is the more useful surrogate, there is a critical need to develop decisive exposure–response information for biologic markers of dose and effect in both laboratory animals and humans. This will help determine whether, with respect to metabolism, genotoxic response, and cancer risk, mice or rats exposed to BD in the laboratory more closely resemble humans exposed to BD in the workplace.

The genotoxic potential of BD has been demonstrated in both rodents and humans, although the species susceptibility to perturbations in various biologic markers of DNA damage differs greatly (Himmelstein et al. 1997; Walker and Meng 2000; Albertini et al. 2003). For example, increased frequencies of chromosomal aberrations, sister chromatid exchanges, and micronuclei were observed in mice exposed to BD, whereas increases in these biomarkers were not observed in similarly exposed rats (Cunningham et al. 1986; Autio et al. 1994; Anderson et al. 1997). Yet BD has been reported to produce increased M*f*s (measured by the T-cell cloning assay) and mutant variants (measured by the autoradiography assay) at the *Hprt* locus of T cells (T lymphocytes) from both exposed rodent species (Cochrane and Skopek 1994b; Tates et al. 1994, 1998; Walker and Meng 2000) as well as from exposed people (Ward et al. 1994, 1996a,b; Ma et al. 2000). Moreover, the increased frequencies of *Hprt* mutants and variants found by Ward and colleagues (1994, 1996a,b) in BD workers in

the United States were associated (1) in an exposure–related manner with a specific BD metabolite in the urine of different populations of exposed workers and (2) with a shift in the spectrum of mutations compared with that of unexposed control subjects (Ma et al. 2000). In molecular epidemiology studies of BD-exposed workers in China (Hayes et al. 1996) and Europe (Tates et al. 1996), consistent exposure-related effects on *HPRT* M*f*s and cytogenetic endpoints have not been observed. Likewise, a recently completed transitional epidemiologic study of BD-exposed Czech workers (Albertini et al. 2003) did not demonstrate any statistically significant correlations between BD exposure and changes in a battery of biomarkers of effect; even conducting the highly sensitive assays to measure *HPRT* M*f*s and variants did not yield positive results. In contrast, certain biomarkers of exposure (hemoglobin adducts and urinary metabolites) were useful measures of BD exposure in worker populations in China and the Czech Republic (Hayes et al. 1996; Swenberg et al. 2001; Albertini et al. 2003). These biomarkers (particularly hemoglobin adducts) may prove to be valuable in future epidemiologic studies of the health effects of BD exposure in humans (Albertini et al. 2003).

Genotoxicity studies of BD in experimental systems indicate that the carcinogenicity of this agent is related to its bioactivation to several DNA-reactive epoxides, including BDO, BDO_2, and BDO-diol (Himmelstein et al. 1997). BD is first metabolized by cytochrome P450 to BDO, which can then be metabolized further via P450 enzymes to BDO_2 or via epoxide hydrolase (EH) to BD-diol; BDO_2 and BD-diol can then be oxidized to BDO-diol (Figure 1; reviewed in Himmelstein et al. 1997). As with ethylene oxide (Golberg 1986), the simplest epoxide, the reactivities of BDO, BDO_2, and BDO-diol are due to the strain of the oxirane ring(s) and the partial charges on the carbon atoms. These properties give BD metabolites their electrophilic character and predispose the ring(s) to open and form carbonium ions. Consequently, BDO, BDO_2, and BDO-diol (1) can be detoxified by EH or by glutathione *S*-transferase (GST; with the glutathione conjugates being excreted in the urine; Figure 1), or (2) can react with nucleophilic sites to form adducts in a variety of cellular macromolecules, including DNA, RNA, and proteins (Swenberg et al. 2001, 2002).

BD-diol is a marker for the flux (1) through the hydrolysis detoxification pathway of BD leading to the major

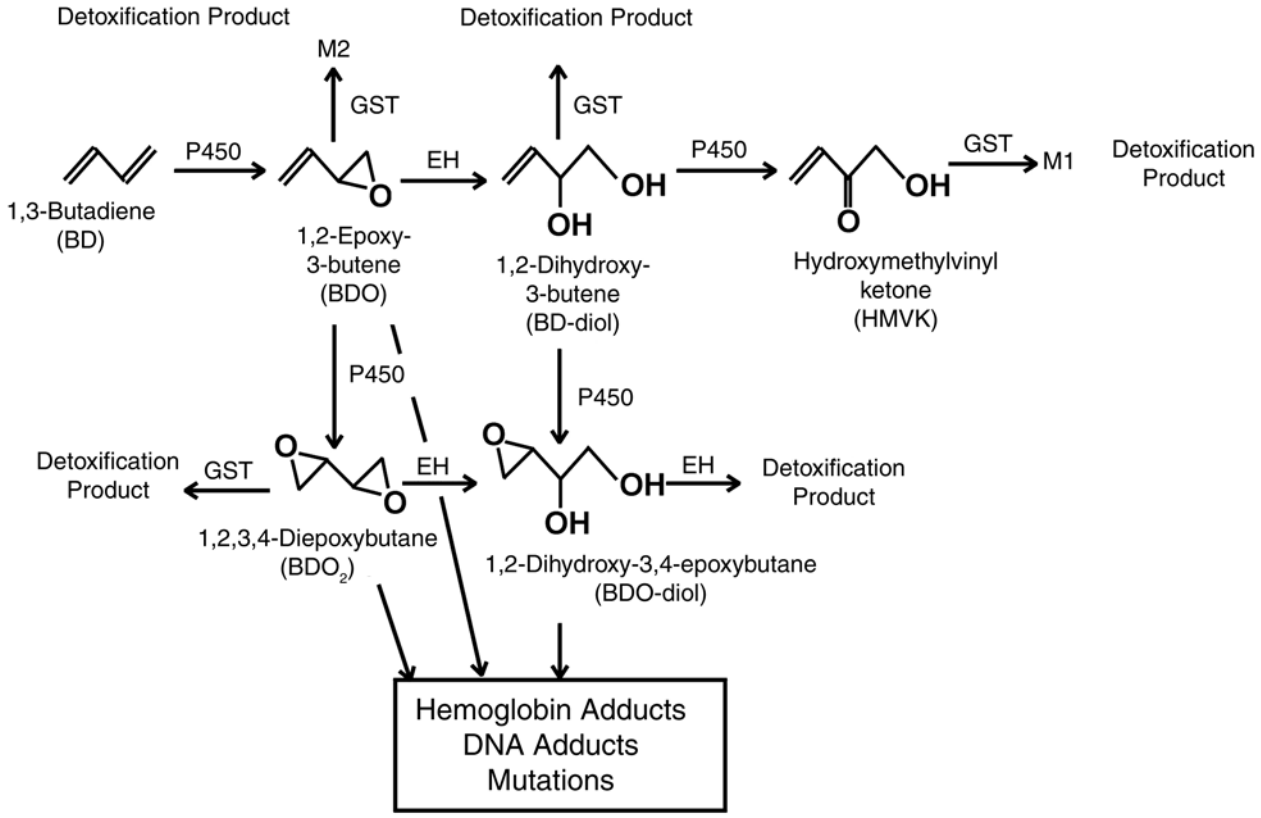

Figure 1. Metabolism of BD. Enzymes involved in BD metabolism. Both M1 and M2 are excreted in urine.

human urinary metabolite 1,2-dihydroxy-4-(*N*-acetylcysteinyl)-butane (M1) or (2) to the oxidative pathway yielding BDO-diol (Figure 1), which is the major source of BD-induced monoadducts in all species studied (Koc et al. 1999; Swenberg et al. 2001, 2002). Hydroxymethylvinyl ketone (HMVK) is a proposed intermediate in the formation of M1 (Figure 1; Krause et al. 2001) and may be another reactive metabolite of BD. (HMVK may also be a spontaneous rearrangement product of 2-hydroxy-3-butenal, formed from BD-diol via alcohol dehydrogenase [Kemper and Elfarra 1996].)

Two of the three epoxy metabolites of BD (BDO and BDO_2) have been measured in tissues of BD-exposed mice and rats. The greater carcinogenic susceptibility of mice correlates with higher circulating concentrations of these genotoxic intermediates (especially BDO_2) compared with those of rats exposed to the same levels of BD (Himmelstein et al. 1994, 1995, 1996; Thornton-Manning et al. 1995a,b, 1996; Elfarra et al. 2001; reviewed in Table 22 of Himmelstein et al. 1997). Different mutagenic potencies of BD metabolites have been demonstrated in vitro, showing that the mutagenic potency of BDO_2 is approximately 100 and 200 times greater than those of BDO and BDO-diol, respectively, in human lymphoblastoid cells that are incapable of further metabolizing the individual epoxides (Cochrane and Skopek 1994a). HMVK is also expected to be mutagenic based on comparisons with other α,β-unsaturated carbonyls. Numerous compounds in this class form promutagenic DNA adducts, specifically 1,N^2-propanodeoxyguanosine adducts, and yield positive results in mutagenicity assays (Benamira et al. 1992; Eder et al. 1993; Burcham and Marnett 1994; Moriya et al. 1994). However, the relative contributions of BDO, BDO_2, and the BD-diol pathways to the mutagenicity and carcinogenicity of BD in mice and rats (the more sensitive and resistant rodent species, respectively) are still uncertain.

The role of stereochemistry is an additional complicating factor that must be considered in the ultimate clarification of species differences in BD-induced mutagenesis and carcinogenesis. Stereochemical considerations arise after the initial oxidation of BD to BDO, because a stereocenter is generated and the chirality of this metabolite may then affect both the subsequent biotransformation pathways (Nieusma et al. 1997) and the formation and persistence of BDO-induced DNA adducts (Koc et al. 1999; Figure 2). In vitro studies with mouse and rat liver microsomes, for example, have demonstrated species-related differences in both the production of BDO and the

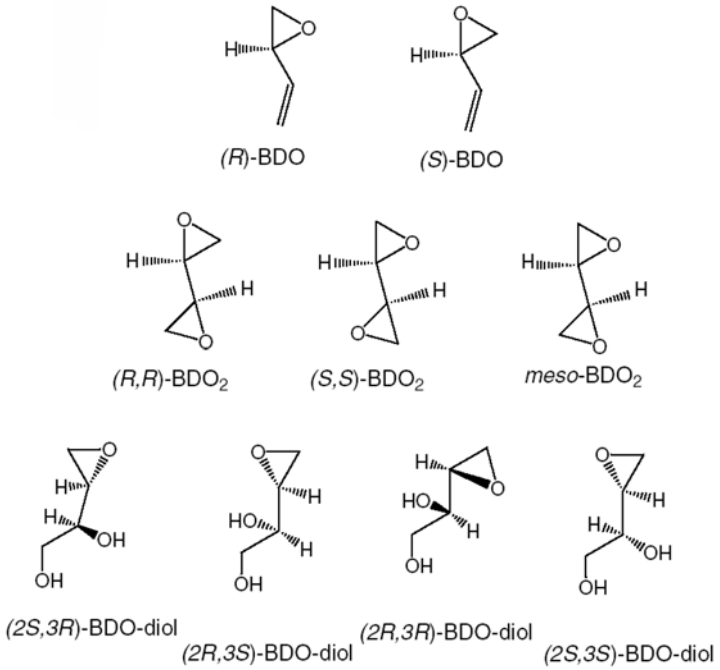

Figure 2. Stereochemistry of BD. In some molecules, the asymmetric bonding of some atoms to a carbon atom causes "left hand" (*S*) and "right hand" (*R*) forms (called stereoisomers) to exist. These forms with asymmetric centers, which are considered mirror images, are chemically identical but differ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

ratio of (*R*)-BDO to (*S*)-BDO (Nieusma et al. 1997). The reasons for these ratios, and for changes in the ratio over time in rats (Nieusma et al. 1997), are not known. But species differences may exist in the interaction of BD with various cytochrome P450 enzymes (Himmelstein et al. 1997) and in the hydrolysis of the enantiomers. BDO is further oxidized in human, rat, and mouse liver microsomes to a mixture of the diastereomers (±)-BDO_2 and *meso*-BDO_2 (Figure 2), in which the relative amounts of each depend on the initial exposure concentration of BDO (Elfarra et al. 1996; Krause and Elfarra 1997). *meso*-BDO_2 appears to be more cytotoxic in vitro than (±)-BDO_2 (Nieusma et al. 1997) but is more readily hydrolyzed in human and rat liver microsomes than in mouse liver microsomes (Krause and Elfarra 1997). These findings demonstrate the potential importance of stereochemistry in BD biotransformation and cytotoxicity and emphasize the need for studies to investigate the contributions of specific stereochemical configurations to the mutagenicity of BD in mice and rats.

In an earlier project sponsored by HEI (Walker and Meng 2000), our research team completed studies attempting to determine (1) whether BD was mutagenic in exposed rats, (2) whether *Hprt* M*f*s in T cells from BD-exposed mice and rats could be correlated with species-related differences in cancer susceptibility, and (3) whether the mutagenic potency and specificity data from mice and rats exposed to BD or its individual epoxy metabolites could reveal the intermediate(s) responsible for mutations in each species. Female B6C3F1 mice were selected as the primary mouse model for mutagenicity studies in that project because (1) tumor data were available for this mouse strain from National Toxicology Program cancer bioassays of BD (Huff et al. 1985; Melnick et al. 1990; Melnick and Huff 1992; U.S. National Toxicology Program 1984) and (2) female mice were more susceptible than male mice, and than both sexes of Sprague-Dawley rats, to the induction of thymic lymphoma and leukemias as well as lung tumors by BD (reviewed in Himmelstein et al. 1997). Although Sprague-Dawley rats were used in carcinogenicity bioassays of BD, female F344 rats were chosen, along with female B6C3F1 mice, for our initial and current mutagenicity studies of BD and its metabolites because (1) the F344 rats and B6C3F1 mice were used in the development of rodent T-cell cloning and molecular analysis methods for *Hprt* mutations (Aidoo et al. 1991; Skopek et al. 1992; Walker and Skopek 1993; Chen et al. 1999; Meng et al. 2004); (2) these strains were used in earlier assessments of the mutagenicity of several chemicals, including BD and its major epoxide metabolites, in preweanling mice and young adult mice and rats (Aidoo et al. 1991, 1993; Skopek et al. 1992; Walker and Skopek 1993; Cochrane and Skopek 1994b; Walker et al. 1996, 1997); and (3) F344 rats were used in carcinogenicity studies of chemical analogues of BD, such as isoprene and chloroprene (U.S. National Toxicology Program 1998, 1999).

Our earlier BD-mutagenicity studies (Walker and Meng 2000) demonstrated that the novel approach of measuring mutagenic potency as the change in *Hprt* M*f*s over time in T cells of exposed animals versus controls was valuable for (1) estimating the species differences in mutagenic response to BD in mice and rats and (2) predicting the potential role of BD-derived metabolites in each species. The resulting *Hprt* M*f* data provided the only experimental evidence to date that BD is mutagenic in rats. Furthermore, the mutagenic response in rats was significantly less than that observed in similarly exposed mice, which was in agreement with the results of cancer bioassays of rodents exposed to BD. The relative contribution of the racemic mixtures of BDO and BDO_2 to the total mutagenicity of BD was investigated by exposing mice and rats to carefully chosen levels of BD, (±)-BDO, and (±)-BDO_2 and assessing the mutagenic potency of each compound when comparable blood concentrations of metabolites were reached.

The resulting *Hprt* M*f* data indicated that at lower exposure levels of BD, (±)-BDO_2 is a major contributor to mutagenic effects in mice, but that other metabolites and stereochemical configurations are likely to be responsible for mutagenic effects in rats and, at higher exposure levels, for incremental mutagenic effects in mice. These initial experiments with BD metabolites were restricted to (±)-BDO and (±)-BDO_2 in large part because in vitro mutagenicity studies in human lymphoblastoid cells had previously demonstrated that the mutagenic potencies of these two racemic mixtures were greater than that of BDO-diol (Cochrane and Skopek 1994b). Thus, assessments of the in vivo mutagenicity of (±)-BDO and (±)-BDO_2 seemed a logical starting point for our investigation of the roles of epoxy metabolites and stereochemistry in the induction of mutations by BD at the *Hprt* locus of rodents.

Comparisons of DNA alkylation and *Hprt* M*f* patterns, reported in collaborative studies supported by HEI (Walker and Meng 2000; Swenberg et al. 2001), provided some evidence for the relative effects of BDO, BDO_2, and BDO-diol in BD-exposed mice. BDO-induced formation of hydroxybutenyl adducts at N7 of guanine (BDO-Gua) and BD-induced *Hprt* mutations showed poor correlations in mice in vivo, suggesting that BDO is not the primary source of BD-induced mutations (Walker and Meng 2000; Swenberg et al. 2001). In contrast, BD-induced formation of trihydroxybutyl adducts at N7 of guanine (THB-Gua) and BD-induced *Hprt* mutations showed highly positive correlations. THB-Gua adducts presumably arise from BDO_2 and

BDO-diol, suggesting the importance of these metabolites in BD-induced mutations in mice. These observations indicate the great need to determine more definitively the relative contributions of BDO_2 and BDO-diol to the mutagenicity of BD at low and high exposures in mice and other species.

One aim of the current project was to expand our understanding of the roles of the three epoxy metabolites and stereochemical configurations in BD-induced mutagenicity by addressing several critical gaps in our knowledge that were revealed while completing recent rodent studies sponsored by HEI (Walker and Meng 2000; Swenberg et al. 2001). Accumulated data suggest potential roles for *meso*-BDO_2 and BDO-diol in BD-induced mutations, and we gave priority to investigating the effects of these BD metabolites in rodent models.

Although much has been learned from earlier experiments that examined the mutagenic and carcinogenic potential of BDO_2, the resulting data may be of limited value because the studies used only the commercially available racemic mixture (Cochrane and Skopek 1994b; Tates et al. 1998; Walker and Meng 2000; Recio et al. 2001). In in vitro studies (Krause and Elfarra 1997), both (±)-BDO_2 and *meso*-BDO_2 appeared to be formed from BDO in mouse, rat, and human liver microsomes, and *meso*-BDO_2 was formed in excess of (±)-BDO_2 in the presence of cDNA-expressed human cytochrome P450 2E1. In addition, *meso*-BDO_2 seemed to be more toxic than (±)-BDO_2 to hepatocytes in vitro (Nieusma et al. 1997). Yet the relative amounts of *meso*-BDO_2 and (±)-BDO_2 formed in vivo in rodents after BD inhalation have not been determined. Recent DNA adduct studies of livers from BD-exposed mice and rats have demonstrated the accumulation of THB-Gua adducts presumably derived from a combination of (±)-BDO_2, *meso*-BDO_2, and BDO-diol (Koc et al. 1999; Oe et al. 1999; Swenberg et al. 2001). The THB-Gua adducts of *meso*-BDO_2 were found to persist longer in mouse liver than in rat liver, and hence it is possible that *meso*-BDO_2 may be more carcinogenic in mice than (±)-BDO_2 (Oe et al. 1999). Lastly, molecular analyses for mutations in *Hprt* cDNA from expanded mutant T-cell clones from control and BD-exposed female mice demonstrated a significant exposure-related increase in the frequency of large deletions ($P = 0.016$), presumably associated mainly with the in situ formation of (±)-BDO_2 and *meso*-BDO_2 (Meng et al. 1999a).

Based on comparisons of exposure–response relations for blood concentrations of BDO_2 and tissue concentrations of THB-Gua adducts in BD-exposed mice and rats, Swenberg and colleagues (2001) concluded that the diastereomers of BDO_2 alone cannot account for the in vivo formation of these adducts and that BDO-diol appears to be the main epoxy metabolite yielding monoadducts in both species. Csanády and coworkers (1996) have suggested that the formation of BD-diol occurs via intrahepatic metabolism in a coordinated manner along the endoplasmic reticulum. A likely source of the large species difference in carcinogenic susceptibility to BD may be the amount of BDO_2 that is formed and escapes immediate hydrolysis and is thus available for DNA cross-linking (Koc et al. 1999). Recent metabolism studies to investigate blood levels of BD, BDO, BDO_2, and BD-diol in BD-exposed mice and rats showed that the dominant circulating metabolite in both species is BD-diol (Filser et al. 2007), the precursor of BDO-diol (as well as of HMVK). An original goal of this project was to determine the relative contribution of BDO-diol to BD-induced mutagenicity in rodents by exposing mice and rats directly to a well-characterized mixture of the stereoisomers of BDO-diol (Figure 2) or to BD at exposure levels that would yield equivalent circulating concentrations of BDO-diol.

Another aim of the current project was to learn more about the mutagenic potency and specificity of BD in mice and rats by exposing them to levels that have been carcinogenic in one or both species and reducing levels to those that approach workplace exposures. The genotoxic effects of BD have been consistently detected in mice, but the exposure levels of BD in our initial studies were much higher than the levels generally encountered in the environment or workplace (Walker and Meng 2000). As a part of this project, mice were exposed to 3 ppm BD, a level that approaches the occupational exposure limit of 1 ppm BD proposed by the U.S. Occupational Safety and Health Administration. The results were compared with earlier exposure–response data obtained for *Hprt* M*f*s and adducts in mice exposed to $\geq$ 20 ppm BD under the same experimental conditions (Meng et al. 2001). Recent studies have demonstrated the occurrence of background concentrations of hemoglobin adducts presumably derived from the endogenous formation of BD in several species (Swenberg et al. 2001), suggesting that there may be threshold levels for the induction of both DNA adducts and mutations associated with exogenous exposure to BD. Therefore, it is important to clarify the exposure–response relations for adducts and mutations at low BD exposures in the more sensitive rodent species (the mouse) for comparisons with biomarker data obtained in studies of BD workers (Hayes et al. 1996; Albertini et al. 2003). These comparisons may ultimately affect the regulation of BD exposures and the assessment of human cancer risk (Albertini 2004).

Characterization of the genetic alterations caused by BD and its reactive intermediates has been used to investigate the nature of DNA adducts leading to mutations and to determine the relative significance of BD metabolites in the mutagenicity of BD. For example, the mutational specificity of BD was characterized in the *lacI* gene in transgenic mice (Recio et al. 1996; Saranko et al. 2001) and in the exon 3 region of the endogenous *Hprt* gene in mice and rats (Cochrane and Skopek 1994a; Meng et al. 2000). Results of these studies have provided several insights into the mechanisms of BD mutagenic actions. However, certain types of mutations that are potentially important in the mutagenicity of BD could not be characterized because of limitations in the experimental systems or molecular analysis techniques used. The Rat2 *lacI* transgenic fibroblast (Rat2 cell) and *lacI* transgenic mouse models, both of which use a shuttle vector system, can define the spectrum of point mutations but not large deletions and chromosomal rearrangements. Similarly, characterization of mutations at *Hprt* exon 3 by denaturing gradient gel electrophoresis analysis is limited to detection of point mutations, small insertions, and small deletions.

Thanks to recent improvements in expanding rodent *Hprt*-negative T-cell clones, mutant genomic DNA and mRNA can now be produced in larger quantities, making it possible to use such techniques as multiplex PCR to evaluate large-scale genomic alterations and RT–PCR to evaluate small-scale mutations in *Hprt* (Chen et al. 1998; Meng et al. 1998b, 1999a). Multiplex PCR analysis of genomic DNA can identify large DNA sequence changes across *Hprt*. A multiplex PCR method for analysis of human *HPRT* gene (Gibbs et al. 1990) has been widely used for over two decades (Albertini 2001). But early difficulties in propagating mutant T-cell clones from rodents hindered the development of multiplex PCR techniques for mouse and rat *Hprt* (Meng et al. 1998b). A rat multiplex PCR method was recently developed and used for the molecular analysis of genomic DNA in *Hprt* mutants from thiopeta-exposed F344 rats (Chen et al. 1999).

The final aim of the current project was to develop a multiplex PCR procedure for mouse *Hprt* and to establish more comprehensive *Hprt* spectra data for large- and small-scale mutations occurring in both BD-exposed and sham-exposed control mice and rats. In general, BD and its epoxy metabolites are relatively weak inducers of gene mutations and better inducers of clastogenic effects (Himmelstein et al. 1997; Walker and Meng 2000); 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. Comparing lymphocyte *Hprt* mutation spectra in BD-exposed humans and rodents may aid in the identification of (1) the metabolite(s) leading to specific types of BD-induced mutations and (2) the more appropriate rodent model for predicting the health effects of BD exposure in humans.

In short, then, the original aims of the current project, which were changed during the course of the work, were (1) to determine the mutagenic potency of *meso*-BDO_2 compared with that previously found for (±)-BDO_2 in similarly exposed mice and rats, (2) to develop a method for measuring BDO-diol in plasma or tissues and to determine the mutagenic potency for BDO-diol–exposed mice and rats compared with earlier estimates of mutagenic potency for animals exposed to BD or other epoxy metabolites, and (3) to determine the relative mutagenic potency and specificity of BD at the *Hprt* locus of male mice and rats.

During the course of the project, several decisions were made that expanded its scope. The first noteworthy change was that, in order to determine the relative contribution of BDO-diol to BD-induced mutagenicity, mice and rats would be exposed to BD-diol (instead of BDO-diol) by direct inhalation at exposure levels that would produce plasma concentrations of BD-diol equivalent to those found after mice were exposed to selected levels of BD. There were several striking advantages to this alternative approach: First, the use of BD-diol would eliminate concerns about the proportions of BDO-diol isomers that would have to be prepared in order to expose animals to a suitable mixture of BDO-diol stereoisomers. This is because exposing mice and rats to BD-diol leads to the formation of BDO-diol as the only epoxy intermediate, and stereoisomers of BDO-diol (Figure 2) are formed in situ in the same ratios as those produced by exposure to BD (Nieusma et al. 1998). Second, measurements of *Hprt* M*f*s (and types of *Hprt* mutations) in mice and rats after in vivo exposure to BD-diol would reflect the combined mutagenic effects of both BDO-diol and HMVK and thus would determine whether the overall mutagenicity of the BD-diol pathway is sufficient to warrant future exploration of the degrees to which BDO-diol and HMVK contribute to mutations in mice versus rats at low to high levels of BD exposure. Third, comparisons between measured blood concentrations of BDO_2, BD-diol, and BDO-diol; concentrations of hemoglobin and DNA adducts; and the mutagenic responses in mice and rats exposed to BD, (±)-BDO_2, *meso*-BDO_2, and BD-diol could be used to derive a differential that could distinguish between the degrees of mutagenicity from BDO-diol that arise from BD-diol versus BDO_2 (Figure 1; Walker and Meng 2000; Swenberg et al. 2001, 2002). Fourth, BD-diol is a stable compound that is commercially available (Chen and Ruth 1993; Cheeseman

et al. 2004), which would eliminate the need for costly synthesis of the BDO-diol stereoisomers.

The second noteworthy change was that we would develop a multiplex PCR procedure for analyzing genomic DNA for mutations in mouse *Hprt* and use a combination of molecular analysis techniques to accomplish (for the first time ever) the coincident (or concurrent) identification of both large- and small-scale genetic alterations across the *Hprt* of BD-exposed and control mice and rats. This change required the use of male mice and rats as sources of *Hprt* mutant T-cell colonies for molecular analyses. (Genomic DNA from female rodents has two *Hprt* alleles and must be analyzed under quantitative assay conditions; genomic DNA from males has one X-linked allele and can be analyzed more efficiently with multiplex PCR using a nonquantitative assay.) Because our previous BD mutagenicity studies, and the rest of the studies planned in the current investigation, all used female rodents, the inclusion of male rodents for exposures to high levels of BD offered the opportunity to assess potential sex differences in mutagenic susceptibility to BD exposure.

A variety of BD-induced mutagenic effects have been observed in experiments using rodents of different strains, sexes, and ages, as well as using various time points for M*f* measurements (Cochrane and Skopek 1994b; Tates et al. 1994, 1998; Meng et al. 1998a, 1999a). Differences in experimental design and observed mutagenic potency of BD in rodents have hindered the direct comparison of experimental results among laboratories and have complicated the application of BD mutagenic effect data to cancer risk assessment of BD exposure in humans (Tates et al. 1994, 1998; Walker and Meng 2000). In order to help overcome these hindrances, we evaluated age and sex as variables that potentially affect mutagenic responses to BD in the T cells of mice and rats.

SPECIFIC AIMS

This project was designed to test the following five hypotheses:

1. Repeated BD exposures at low levels that approach the occupational exposure limit for BD workers (set by the U.S. Occupational Safety and Health Administration) are mutagenic in female mice.
2. The differences in mutagenic responses of the *Hprt* gene to BD in similarly exposed rodents of a given species (reported in various earlier studies) are primarily associated with age-related thymus activity and trafficking of T cells and with sex-related differences in BD metabolism.
3. The mutagenic potency of the stereochemical forms of BD's epoxy intermediates plays a significant role in the species-related mutagenicity of BD.
4. The metabolic pathway of BD through BD-diol is a major contributor to mutagenicity at high-level BD exposures in mice and rats.
5. Significant and informative species-specific differences in mutation spectra can be identified by analyzing both large- and small-scale genetic alterations in the *Hprt* gene of BD-exposed mice and rats.

These hypotheses were tested by conducting experiments to address each of five specific aims.

Specific Aim 1. Low-Level BD Experiments *Hprt* M*f*s in female mice exposed to low-level BD would be determined, and the resulting data would be combined with those obtained previously to further define the shape of the exposure–response curve for M*f*s in mice exposed over a wide range of 3 to 1250 ppm BD.

Specific Aim 2. Age- and Sex-Related BD Experiments Age- and sex-related mutagenic responses in mice and rats exposed to high levels of BD (0 or 1250 ppm) would be determined and compared with results from earlier studies.

***Specific Aim 3.* meso-*BDO_2* Experiments** The mutagenic potency of *meso*-BDO_2 in exposed mice and rats would be determined for comparison with previously obtained estimates of mutagenic potency in mice and rats exposed to (±)-BDO_2.

Specific Aim 4. BD-diol Experiments Techniques for measuring BD-diol in plasma would be developed, and the relative contribution of BD-diol–derived metabolites to BD-induced mutagenicity would be determined by evaluating (1) the effects of the time elapsed after exposure and (2) the effects of exposure level on *Hprt* M*f*s in splenic T cells from mice and rats exposed directly to BD-diol (rather than indirectly via exposure to BD).

Specific Aim 5. BD Mutation-Spectra Experiments A multiplex PCR method would be developed for analyzing genomic DNA to identify mutations in the mouse *Hprt* gene. (For rats, we would adapt and improve an existing method.) Multiplex PCR methods would be used in combination with RT–PCR and cDNA sequencing for the coincident identification of both large- and small-scale genetic alterations occurring across the *Hprt* genes of control mice and rats compared with mutant clones from male mice and rats exposed to BD for Specific Aim 2.

METHODS AND STUDY DESIGN

CHEMICALS

BD (more than 99% pure) and BD-diol (racemic mixture, more than 99% pure) were obtained commercially from Sigma-Aldrich (St Louis, MO). *meso*-BDO_2 was custom synthesized as described below for Specific Aim 3. The following cell-culture reagents were purchased: 6-thioguanine (6-TG) and β-mercaptoethanol from Sigma-Aldrich; concanavalin A from Worthington Biochemical (Lakewood, NJ); Lympholyte M and R from Accurate Chemical & Scientific (Westbury, NY); HEPES buffer from Fisher Scientific (New York, NY); HL-1 medium from BioWhittaker (Walkersville, MD); and rat T-STIM culture supplement and mouse recombinant interleukin-2 from Collaborative Biomedical Products (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, at the NY State Department of Health for Specific Aim 1. The same reagents were obtained from BioWhittaker for experiments in Specific Aims 2 through 4. Reagents used for monitoring *meso*-BDO_2 and BD-diol atmospheres and for analyzing BD-diol, M1, and M2 [an isomeric mixture of the regio- and stereoisomers 1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene and 2-hydroxy-1-(*N*-acetylcysteinyl)-3-butene; see Figure 1] were the highest grades commercially available; the sources for these are given below for Specific Aims 3 and 4. Other chemicals and reagents used for total RNA or genomic DNA extraction, PCR amplification, evaluation of PCR efficiency, and DNA sequencing are given below for Specific Aim 5.

ANIMALS AND HUSBANDRY

Female and male B6C3F1 mice (20 to 22 days old, or 6 weeks ± 2 days old for one group of females) were obtained from the Charles River Laboratories facility in Raleigh, North Carolina, or Portage, Michigan. Male and female F344 rats (20 to 22 days old) were obtained from the Charles River Laboratories facility in Hollister, California. All animals were free of virus titers, as determined by standard mouse and rat virus antibody assays (Microbiologic Associates, Bethesda, MD). They were acclimated for approximately 2 weeks before starting chemical exposures. The housing of rodents conformed to federal guidelines (U.S. National Research Council 1996). Animals were randomly separated into exposure and control groups by weight and were housed individually in hanging wire stainless-steel cages in rooms controlled for temperature (72 ± 4°C) and humidity (50 ± 10%) with a 12-hour light/dark cycle. In the mutagenicity studies reported here, a number of additional mice and rats were added to the study groups and exposed in order to collect blood and tissues to be used for measurements of hemoglobin and DNA adducts derived from BD metabolites in future studies of BD-induced carcinogenesis. All procedures using animals were approved by the Institutional Animal Care and Use Committees at the Wadsworth Center and Lovelace Respiratory Research Institute, where the animal experiments were performed.

ISOLATION AND CULTURE OF RODENT *Hprt* MUTANT T CELLS

The general procedures for isolating T cells from mouse and rat spleen and culturing *Hprt* mutant T-cell colonies for chemical mutagenic potency and specificity studies have been described in detail elsewhere (Skopek et al. 1992; Walker and Skopek 1993; Meng et al. 1998a,b). Briefly, spleens were removed from mice and rats aseptically, and T cells were isolated by macerating spleens individually in 12-well microtiter dishes, layering the cells on a Ficoll gradient (Lympholyte M or R), and washing the recovered cells with RPMI-1640 medium. The cells were then resuspended in priming medium for mitogenic stimulation with concanavalin A overnight.

For the current investigation, priming medium and cloning medium for the stimulation and growth of mouse T-cells were modified by the addition of 10% rat T-STIM, a conditioned medium from concanavalin-A–stimulated rat splenocyte cultures (Meng et al. 1998b). Rat T cells were stimulated and grown in the same media without rat T-STIM. After overnight priming, cells were counted using a Coulter Counter (model 2BI, Beckman Coulter, Fullerton, CA) and cultured in 96-well U-bottom microtiter plates with supplemented medium (Meng et al. 1999a) to determine cloning efficiencies (CEs) and to identify *Hprt* mutants. To measure CEs for T cells, aliquots of primed cultures at 4×10^5 cells/mL were diluted with cloning medium so that an average of eight mouse or four rat splenic T cells per well were cultured in the presence of lethally irradiated mouse or rat splenic T cells (feeder cells; 2×10^4/well). To isolate *Hprt* mutants, primed cultures were diluted to 4×10^5 cells/mL with cloning medium supplemented with 1 µg 6-TG/mL and then seeded in 96-well plates at 100 µL medium/well for incubation. Plates were scored for colony growth using a magnification of 40× (and confirmed at higher magnifications as necessary) on days 8 and 9.

Hprt M*f*s were calculated as described below (Skopek et al. 1992). M*f* data for individual exposure and control

groups, different chemicals, and different exposure levels were expressed as the mean M*f* ± SD. CE was calculated using the Poisson distribution, which allows one to estimate the average number of colony-forming units per well (λ). Because the probability of observing a negative well (i.e., a well with no colony growth) is $P(0) = e^{-\lambda}$ [λ estimated using −ln (fraction of observed negative wells)], the CE of a culture is estimated as −ln *P*(0)/number of cells per well (Skopek et al. 1992). As shown below, M*f* was calculated as the ratio of the mean CE in selective medium to that in nonselective medium.

$$Mf = \frac{-\ln\left[\dfrac{\text{negative wells on selection plates}}{\text{total wells}}\right]}{(\text{CE on nonselection plates})\left(\dfrac{\text{cells plated}}{\text{well}}\right)}$$

SUMMARY OF EXPERIMENTAL PROTOCOLS AND EXPOSURES

The methods and study designs presented below were fairly complex. Table 1 presents an overview for the first four Specific Aims, which involve exposures. The table includes the exposure compound and regimen, age and sex and species of the animals exposed, times after exposure for necropsy, endpoints measured, and the sources of comparison data.

Changes to the Planned Protocol

In the original project design, all animals for Specific Aims 1 and 2 were to be exposed to BD concurrently (for 2 weeks, 6 hours/day, 5 days/week). That is, 4- to 5-week-old female mice would be exposed to 0 or 3 ppm BD for Specific Aim 1; and 8- to 9-week-old female mice and 4- to 5-week-old male mice and rats would be exposed to 0 or 1250 ppm BD for Specific Aim 2. (The animals from Specific Aim 2 would provide splenic T cells for analysis in Specific Aim 5.) The female mice exposed to 0 or 3 ppm BD for Specific Aim 1 were analyzed as planned (see the next section).

Daily monitoring of high-level exposures, however, showed that because of technical problems, the 1250-ppm exposure groups were inadvertently exposed to approximately 3500 ppm BD for the first 3 of 10 exposure days. When the problem was noted, we decided to continue the high-level exposure at 1000 ppm BD for the next 7 days because of the large numbers of mice and rats included in the chambers for Specific Aim 2 (studies of sex- and age-related differences in the mutagenic potency of BD). This modified exposure regimen resulted in an average exposure level of approximately 1750 ppm BD for the 2-week inhalation period. Because the target level was 1250 ppm, we conducted an additional inhalation exposure of male and female mice and male rats to 0 or 1250 ppm BD to address Specific Aims 2 and 5. The animals exposed to nominal 1750 ppm were analyzed for *Hprt* M*f*s and those results have been published elsewhere (Meng et al. 2001).

SPECIFIC AIM 1. LOW-LEVEL BD EXPERIMENTS

Female mice were exposed to 0 or 3 ppm BD by inhalation using multitiered whole-body exposure chambers (model H-2000; Lab Products, Seaford, DE). The total volume of each chamber was 1.7 m^3. The flow rate through each chamber was maintained at 15 ± 2 air changes per hour. All air was HEPA-filtered before being introduced into the chamber supply system. Exposures were conducted using BD gas delivered from compressed gas cylinders to each chamber. Flow from each cylinder was controlled via a rotameter. Rodents in the control chamber were sham-exposed to filtered air only. Each exposure day lasted for 6 hours + T_{90} (time to reach 90% of the target level), which was determined before the start of exposure for each level. Animals were housed in the exposure chambers throughout the study and had free access to food (NIH-07 certified feed) and water except during exposure periods, when food was removed.

Vapor concentrations in exposure chambers were monitored by two independent methods. In the first, an infrared spectrometer (model MIRAN IA; Foxboro Company, Foxboro, MA) was used to monitor chamber vapor concentration periodically in real time via infrared absorbance. The MIRAN IA is a single-beam, variable-filter infrared spectrometer; the gas cell parameters, wavelength, and path length were adjusted for optimal operation. In the second method, grab samples were collected three times during each exposure day and analyzed by GC.

BD atmospheres were compared with five-point calibration curves that span the exposure range required to accurately measure the lower and upper ends of the BD exposure range used in these rodent inhalation studies (for both Specific Aims 1 and 2). At no time did the BD exposure atmosphere exceed the upper calibration concentration. Analysis of BD exposure atmospheres by GC and a flame ionization detector (FID; model 300H; California Analytical Instruments, Irvine, CA) revealed no impurities, attesting to both the stability of the exposure atmosphere and the purity of the reference BD (certified as more than 99% pure).

Table 1. Overview of Exposure Protocols for Specific Aims 1 Through 5 and Sources of Comparison Data[a]

Exposure Compound and Regimen	Sizes of Exposure Groups and Times of Necropsy				Goals, Analyses, and Sources of Comparison Data from This and Other Studies
	Female Mice (*n*)	Female Rats (*n*)	Male Mice (*n*)	Male Rats (*n*)	
Aim 1. Low-Level BD Experiments					
BD 0 or 3 ppm 2 weeks	0 ppm 24 3 ppm 27 Necropsy 4 weeks				Combined current data with earlier data to define the shape of the exposure–response curve for *Hprt* M*f*s in mice exposed to between 3 and 1250 ppm BD: • For M*f*s, added to earlier data (Meng et al. 1999a; Walker and Meng 2000) to expand the BD exposure range to as low as 3 ppm. • For CEs, compared with data from mice of the same age exposed to 20–1250 ppm BD (Meng et al. 1998a, 1999a; Walker and Meng 2000). • For M*f*–CE relation, compared with data from mice exposed to 20 ppm BD (Meng et al. 1999a; Walker and Meng 2000) and with data from earlier studies of humans (Cole et al. 1980; Tates et al. 1991).
Aim 2. Age- and Sex-Related BD Experiments and **Aim 5. Mutation Spectra Experiments**					
BD 0 or 1250 ppm 2 weeks	8–9 weeks old 0 ppm 25 1250 ppm 22 Necropsy 4 or 5/group at 0 (controls only), 2, 5, 8, 11, or 13 weeks		4–5 weeks old 0 ppm 11 1250 ppm 30 Necropsy 5 weeks	4–5 weeks old 0 ppm 5 1250 ppm 15 Necropsy 4 weeks	Compared mutagenic responses and *Hprt* M*f*s in splenic T cells with those from similarly exposed mice and rats in earlier studies (Meng et al. 1998a; Walker and Meng 2000). • For age-related differences, compared female mice 8 to 9 weeks old from this study with female mice 4 to 5 weeks old from earlier studies; compared the patterns of the mutant manifestation curves across time after exposure for each age group. • For sex-related differences, compared male mice and rats with female mice and rats of the same age from earlier studies. Collected mutant clones from male mice and rats and expanded them for molecular analyses in Specific Aim 5.

Table continues next page

[a] All animals were exposed at 4 to 5 weeks old, except for one group of female mice at 8 to 9 weeks of age for Specific Aim 2. Single 6-hour exposures were nose-only. Multiweek exposures were in inhalation chambers for 6 hours/day, 5 days/week. Necropsy times are after cessation of exposure.

Table 1 (*Continued*). Overview of Exposure Protocols for Specific Aims 1 Through 5 and Sources of Comparison Data[a]

Exposure Compound and Regimen	Sizes of Exposure Groups and Times of Necropsy				Goals, Analyses, and Sources of Comparison Data from This and Other Studies
	Female Mice (*n*)	Female Rats (*n*)	Male Mice (*n*)	Male Rats (*n*)	
Aim 3. *meso*-BDO_2 Experiments					
meso-BDO_2 0 or 2 ppm 3 weeks	0 ppm 27 2 ppm 25 Necropsy 4 or 5/group at 1, 2, 4, 5, 6, or 7 weeks	0 ppm 30 2 ppm 30 Necropsy 4 or 5/group at 1, 2, 4, 5, 6, 7, or 9 weeks			*3-Week Exposures* Compared the effects of time after exposure to *meso*-BDO_2 on mutagenic potency (*Hprt* M*f*s) for exposed female mice and rats with previously obtained estimates for mice and rats exposed to (±)-BDO_2 (Meng et al. 1999b; Walker and Meng 2000).
meso-BDO_2 0, 2, or 4 ppm 2 weeks	0 ppm 5 2 ppm 6 4 ppm 6 Necropsy 4 weeks	0 ppm 5 2 ppm 5 4 ppm 5 Necropsy 4 weeks	0 ppm 7 2 ppm 8 4 ppm 8 Necropsy 5 weeks	0 ppm 5 2 ppm 6 4 ppm 6 Necropsy 5 weeks	*3-Week and 2-Week Exposures* Assessed CEs for female animals from both the 3-week and 2-week exposures. *2-Week Exposures* Examined effects of exposure level on *Hprt* M*f*s to identify sex- and species-related differences. Collected mutant clones from male mice and rats for future molecular analyses.
Aim 4. BD-diol Experiments					
Inhalation Study A BD 0, 62.5, 625, or 1250 ppm 6 hours	6/level Necropsy 0 hour	5/level Necropsy 0 hour	6/level Necropsy 0 hour	5/level Necropsy 0 hour	**BD-diol in plasma after exposure to BD or BD-diol** Compared results of Inhalation Studies A and B to: • Identify BD-diol exposure levels that would yield the same BD-diol plasma concentrations as those resulting from exposures to 62.5 and 625 ppm BD. • Examine how species, sex, and exposure level affect plasma concentrations of BD-diol. • Test the sensitivity of the analytic method adapted to measure BD-diol. Collected tissues for future studies.
Inhalation Study B BD-diol 0, 6, or 18 ppm 6 hours	6/level Necropsy 0 hour	6/level Necropsy 0 hour	6/level Necropsy 0 hour	6/level Necropsy 0 hour	

Table continues next page

[a] All animals were exposed at 4 to 5 weeks old, except for one group of female mice at 8 to 9 weeks of age for Specific Aim 2. Single 6-hour exposures were nose-only. Multiweek exposures were in inhalation chambers for 6 hours/day, 5 days/week. Necropsy times are after cessation of exposure.

Table 1 (*Continued*). Overview of Exposure Protocols for Specific Aims 1 Through 5 and Sources of Comparison Data[a]

Exposure Compound and Regimen	Sizes of Exposure Groups and Times of Necropsy				Goals, Analyses, and Sources of Comparison Data from This and Other Studies
	Female Mice (*n*)	Female Rats (*n*)	Male Mice (*n*)	Male Rats (*n*)	
Aim 4. BD-diol Experiments (*Continued*)					
Inhalation Study C					**BD-diol in plasma and *Hprt* Mfs after extended direct exposure to BD-diol (see also Inhalation Study F)**
BD-diol 0, 6, or 18 ppm 4 weeks	31/level Necropsy 6/level 0 week; 5/level at 2, 4, 5.5, 7, or 8 weeks	31/level Necropsy 6/level 0 week; 5/level at 2, 4, 5.5, 7, or 8 weeks	16/level Necropsy 6/level 0 week; 5/level at 4 or 5 weeks	16/level Necropsy 6/level 0 week; 5/level at 4 or 5 weeks	Determined concentrations of BD-diol in plasma and assessed how species and sex affect them. Assessed mutagenicity and *Hprt* Mfs in splenic T cells. Collected mutant clones from male mice and rats for future molecular analyses.
Inhalation Study D					**BD-diol in plasma and urine and M1 and M2 in urine**
BD 0 or 200 ppm 6 hours	30/level Necropsy 6/level at 0, 2, 4, or 6 hours; or after 24 hours for urine collection	30/level Necropsy 6/level at 0, 2, 4, or 6 hours; or after 24 hours for urine collection			Immediately after exposure, determined concentrations of BD-diol in plasma and compared them with those for female mice and rats exposed to 6 or 18 ppm BD-diol [see Inhalation Study B]. Determined BD-diol half-life in plasma. Examined whether BD-diol is excreted unmodified in urine. Compared M1 and M2 in urine with BD-diol in urine and in plasma.
Inhalation Study E					**BD-diol in plasma**
BD-diol 0, 24, or 36 ppm 6 hours	6/level Necropsy 0 hour				Compared concentrations of BD-diol in plasma with those from BD-exposed mice and rats in Inhalation Studies A, B, and D.

Table continues next page

[a] All animals were exposed at 4 to 5 weeks old, except for one group of female mice at 8 to 9 weeks of age for Specific Aim 2. Single 6-hour exposures were nose-only. Multiweek exposures were in inhalation chambers for 6 hours/day, 5 days/week. Necropsy times are after cessation of exposure.

Table 1 (*Continued*). Overview of Exposure Protocols for Specific Aims 1 Through 5 and Sources of Comparison Data[a]

Exposure Compound and Regimen	Sizes of Exposure Groups and Times of Necropsy				Goals, Analyses, and Sources of Comparison Data from This and Other Studies
	Female Mice (*n*)	Female Rats (*n*)	Male Mice (*n*)	Male Rats (*n*)	
Aim 4. BD-diol Experiments (*Continued*)					
Inhalation Study F					***Hprt* M*f*s after extended direct exposure to BD-diol (see also Inhalation Study C)**
BD-diol 0 or 36 ppm 4 weeks	5/level Necropsy 5/level at 2, 4, 5, 6, or 8 weeks	5/level Necropsy 5/level at 2, 4, 5, 6, or 8 weeks	5–8/level Necropsy 5–8/level at 2, 4, 5, 6, or 8 weeks		Measured *Hprt* M*f*s after extended direct exposure to this higher level of BD-diol. Compared current data with those from Inhalation Study C and with those from earlier studies of mice and rats exposed to BD (Walker and Meng 2000). Extended the exposure–response curve for *Hprt* M*f*s induced via the BD-diol pathway. Collected mutant clones from male mice and rats for future molecular analyses.

[a] All animals were exposed at 4 to 5 weeks old, except for one group of female mice at 8 to 9 weeks of age for Specific Aim 2. Single 6-hour exposures were nose-only. Multiweek exposures were in inhalation chambers for 6 hours/day, 5 days/week. Necropsy times are after cessation of exposure.

In accordance with the original study design, female mice exposed to 3 ppm BD (n = 27) and sham-exposed control mice (n = 24) were killed by CO_2 asphyxiation 4 weeks after the cessation of 2 weeks of BD exposure. Their spleens were aseptically removed for isolation of splenic T cells and measurement of *Hprt* M*f*s.

SPECIFIC AIM 2. AGE- AND SEX-RELATED BD EXPERIMENTS

Female mice (8–9 weeks of age) and male mice and rats (4–5 weeks of age) were exposed to 0 or 1250 ppm of BD for 2 weeks (6 hours/day, 5 days/week); animal exposures and atmosphere monitoring were conducted as described above for Specific Aim 1.

To determine changes in M*f* over time after exposure, the 8- to 9-week-old female mice (control and exposed) were killed by CO_2 asphyxiation at 0 (controls only), 2, 5, 8, 11, or 13 weeks after exposure (n = 4 or 5/group/time point, totaling 25 control and 22 exposed female mice). Splenic T cells were isolated, *Hprt* mutant T-cell manifestation curves were plotted, and mutagenic potency was estimated. These data for 8- to 9-week-old female mice were compared with those obtained in earlier studies in which female mice of 4 to 5 weeks of age were similarly exposed to BD (Meng et al. 1998a; Walker and Meng 2000).

To determine sex-related changes in M*f*, 4- to 5-week-old BD-exposed male mice and rats and their respective control groups (30 exposed and 11 control mice, 15 exposed and 5 control rats) were killed as described above. Splenic T cells were isolated, and the resulting *Hprt* M*f*s were measured 5 weeks (for mice) and 4 weeks (for rats) after exposure, which is the time after 2 weeks of such exposure that maximum M*f*s are reached in splenic T cells of animals of this age (Meng et al. 1998a; Walker and Meng 2000). The *Hprt* M*f* data were then compared with those obtained in earlier studies with similarly exposed female mice and rats of the same age (Meng et al. 1998a; Walker and Meng 2000).

SPECIFIC AIM 3. *meso*-BDO_2 EXPERIMENTS

Stereo-Selective Synthesis of *meso*-BDO_2

Custom synthesis of multigram amounts of *meso*-BDO_2 was required for completing Specific Aim 3 because this stereo-specific compound was not available commercially. Large-scale amounts of the chemical were needed for assessing the exposure–response pattern for *Hprt* M*f*s in female mice and rats exposed by inhalation to *meso*-BDO_2, and for comparing the resulting data to those reported in female mice and rats exposed to (±)-BDO_2 (Meng et al. 1999b).

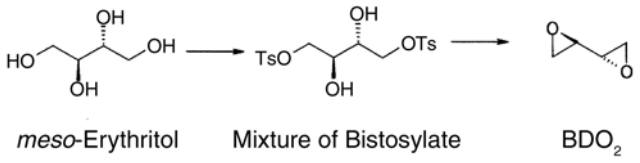

Figure 3. Scheme for the synthesis of *meso*-BDO_2. BDO_2 is ~95% *meso*-BDO_2 and 5% (±)-BDO_2.

A scheme for the synthesis of *meso*-BDO_2, as described briefly below and shown in Figure 3, has been discussed in detail by Claffey (2002). A solution of *meso*-erythritol (100 g, 0.82 mol) in pyridine (3000 mL) was cooled to 5°C by magnetic stirring. Tosyl chloride (296 g, 1.56 mol) was added in stages over 4 hours while maintaining a temperature of 5 to 7°C. The resulting clear solution turned opaque 30 minutes after the last portion of tosyl chloride was added. This solution was stirred for 12 hours at 3 to 5°C and was then allowed to warm to room temperature. The resulting thick suspension was evaporated under reduced pressure at 50°C to yield an orange syrup (approximately 500 mL). These steps resulted in a mixture of bistosylate in good yield and adequate purity along with small amounts of mainly mono-, tri-, and tetratosylate. (Addition of larger amounts of tosylate chloride to the starting solution generally reduced the yield.) The mixture did not require any further purification because the by-products would ultimately yield nonvolatile products when reacted with potassium hydroxide (see step below). The syrup was dissolved in ethyl acetate (2000 mL); 5M hydrochloric acid (HCl) was added one drop at a time with vigorous magnetic stirring until the solution became acidic. The solution was poured into a separation funnel, and the organic portion was removed and washed once with distilled water (200 mL). The ethyl acetate solution was dried over magnesium sulfate, filtered, and evaporated under reduced pressure to yield crude bistosylate as a thick, orange liquid (223 g). The final reaction step was safely carried out by suspending the crude bistosyl mixture in dichloromethane (1500 mL) and gradually adding two equivalents of freshly powdered potassium hydroxide (56 g, 1 mol) over 30 minutes with vigorous magnetic stirring. The mixture was then stirred for 2 additional hours until thin-layer chromatography (silica; ethyl acetate:hexanes 1:1) showed that no bistosylate remained (retention factor of 0.5). The suspension was filtered and the solid was washed with dichloromethane. The dichloromethane was allowed to evaporate in a fume hood (under a dry-ice trap) to yield an orange liquid. The liquid was distilled under reduced pressure with vigorous stirring using a fractionating column. *meso*-BDO_2 distilled as a clear liquid between 26 and 28°C (30 mm Hg; 26 g, 37% yield).

The resulting material was characterized by ^{1}H NMR (nuclear magnetic resonance; 300 MHz, $CDCl_3$, δ): 2.57 (2H, m), 2.70 (2H, m), 2.86 (2H, m); and by ^{13}C NMR (75 MHz, $CDCl_3$, δ): 44.5, 50.42. Its diastereomeric purity (95%) was determined by integration of the ^{13}C signals of the *meso*-BDO_2 with those of the diastereomeric (±)-BDO_2 at δ 43.9 and 50.9 ppm. The identities of the signals of the diastereoisomers were confirmed by comparison with an NMR spectrum of (±)-BDO_2 (Aldrich Chemical Co., Milwaukee, WI). In addition, reaction of the *meso*-BDO_2 synthesis product with calf thymus DNA resulted in the formation of BDO_2-induced N7–N7 guanine cross-link adducts at a ratio of 95 *meso*-specific:5 *racemic*-specific 1,4-bis(guan-7-yl)-2,3-butanediol quantified by GC/MS analysis (Park and Tretyakova 2004). Much of the (±)-BDO_2 may have been produced as a result of the reaction of the by-product, 1,3-*O*-tosyl-erythritol, with potassium hydroxide. (If higher diastereomeric purity was required, the crude bistosylate could have been further purified by recrystallization before reaction with the base.) The *meso*-BDO_2 was stored at −20°C.

Exposure Protocols and Atmosphere Monitoring

Mice and rats (both sexes, 4 to 5 weeks of age) were exposed by inhalation to *meso*-BDO_2 vapor using three multitiered whole-body exposure chambers (model H-1000; Lab Products); rodents in the control chamber were exposed to filtered air only. Otherwise, animal exposures were conducted in the protocol described above for BD exposures. (See below for specific protocols, sexes, species, and times of necropsy.)

To monitor exposures, chamber atmosphere samples were collected in 125-mL glass impingers containing 100 mL of ethyl acetate (analytical grade; Aldrich Chemical). Impingers were placed on ice, and samples were collected at a flow rate of 2 L/min for 60 minutes. After collection, samples were immediately analyzed using GC/MS as described below. Stock standards were made by diluting gravimetrically determined *meso*-BDO_2 into ethyl acetate. Calibration solutions for generating five-point calibration curves spanning the range of *meso*-BDO_2 concentrations used for these rodent inhalation exposures were created using serial dilutions of the stock solution.

Splitless injections (1 µL) were made into a GC/MS (model 5890 GC; model 5970 MS; Hewlett Packard, Palo Alto, CA) equipped with a phenylmethylsilicone cross-bonded fused-silica capillary column (30-mm, 0.25-mm × 0.25-mm; RTX-200MS; Restek, Bellefonte, PA). Operating conditions were as follows: 50°C for 1.0 minute; heat 20°C/min to 130°C; hold 0.5 minutes. Analysis was conducted by selective ion monitoring of ions 31 and 55. The limit of quantitation was less than 100 ng/mL, well below the amounts measured during the monitoring periods.

Protocols to Assess Effects of Exposure Level and Time After Exposure

To define species-related changes in *Hprt* M*f*s over time after exposure, the experimental plan called for female mice and rats to be exposed to 0 or 2 ppm *meso*-BDO_2 for 4 weeks (6 hours/day, 5 days/week). However, the planned 4-week exposure had to be reduced to 3 weeks because the quantity of *meso*-BDO_2 was insufficient to complete a fourth full week.

The exposed and control groups were killed (by CO_2 asphyxiation followed by exsanguination via cardiac puncture) at 1, 2, 4, 5, 6, 7, and 9 (female rats only at this time) weeks after cessation of exposure (n = 4 or 5 of each species/group/time point, totaling 27 control and 25 exposed female mice, and 30 control and 30 exposed female rats) for isolation of splenic T cells.

To determine the exposure–response pattern for *Hprt* M*f*s at a single time point after exposure, groups of female and male mice and rats were exposed for only 2 weeks to 0, 2, or 4 ppm *meso*-BDO_2. The females were necropsied at 4 weeks after exposure and the males at 5 weeks (n = 5 to 8 of each sex/species/exposure level). Splenic T cells were isolated, M*f*s were measured, and mutant colonies were collected from male mice and rats for future molecular studies.

SPECIFIC AIM 4. BD-DIOL EXPERIMENTS

In rodent studies that assess blood levels of BD metabolites, the decision to measure circulating concentrations of BD intermediates in whole blood or plasma depends on the goals of the given experiment and the analytical methods to be applied. In previous studies of BD metabolites, whole blood was typically collected from mice and rats while they were still in place after nose-only exposures so that blood could be frozen immediately to capture peak circulating concentrations of the metabolites under investigation (Thornton-Manning et al. 1995a,b, 1996). Instead, we separated the plasma and red blood cells so that circulating levels of BD-diol and of hemoglobin adducts could be measured independently in the same control and exposed animals. Rather than determining peak circulating concentrations of BD-diol in animals exposed to BD or BD-diol, these experiments were designed to determine (1) the relations between BD and BD-diol exposure levels, (2) BD-diol concentrations in plasma at the selected time interval after exposure (3 minutes), and (3) the magnitude of mutagenic responses resulting from the exposures.

Mice and rats were exposed to either BD or BD-diol to determine for each species the BD-diol exposure levels that would produce the same BD-diol plasma concentrations as those resulting from exposures to 62.5 and 625 ppm BD — concentrations of BD used in previously reported carcinogenicity and mutagenicity studies in mice (Melnick et al. 1990; Melnick and Huff 1992; Walker and Meng 2000).

Exposure Protocols and Atmosphere Monitoring

Male and female mice and rats (4 to 5 weeks of age) were exposed to either BD or BD-diol vapor by inhalation via nose only or in inhalation chambers (model H-2000); rodents in control groups were sham-exposed to filtered air. Whole-body animal exposures were conducted in the protocol described for BD exposures in Specific Aim 1. (See below for specific protocols, sexes, species, and times of necropsy; see also Table 1.)

BD exposure atmospheres for Inhalation Studies A and D were monitored as described for Specific Aim 1; BD-diol exposure atmospheres for Inhalation Studies B, C, E, and F were monitored using two techniques: a real-time hydrocarbon analyzer; and a set of integrated samples to verify the response of the analyzer compared with BD-diol standards of known concentrations. The hydrocarbon analyzer was a real-time FID (model 300H; California Analytical Instruments, Orange, CA) calibrated against a certified propane standard. This instrument gave real-time data for maintaining and measuring changes in the BD-diol chamber concentrations. The integrated samples were collected in 30-mL glass impingers containing 20 mL of purified water. The impingers were placed on ice, and the samples were collected at a flow rate of 0.3 L/min for 30 minutes. Back-up samples were collected periodically to verify the absence of sample break-through in the front trap. Immediately after collection, the samples were analyzed by GC and FID as described below. Stock standards were made from aliquots of the BD-diol purchased for animal exposures. Calibration solutions from 7.8 to 125 µg/mL were created using purified (double-distilled) water. BD-diol atmospheres were compared with five-point calibration curves that spanned the range required to accurately measure the BD-diol concentrations used in our rodent inhalation exposure studies. At no time did the BD-diol exposure atmosphere exceed the highest calibration concentration.

Analysis of exposure atmospheres by GC and FID revealed no impurities, attesting to both the stability of the exposure atmosphere and the purity of the reference BD-diol (certified as more than 99% pure). Splitless injections (1 µL) were made into a GC–FID instrument (model 5890 GC; Hewlett Packard, Palo Alto, CA) equipped with a Stabilwax column (0.53 mm ID, 60 mm length, 1.0 µm degrees of freedom; Restek). The GC oven temperature was not ramped; it was maintained at 200°C over the course of the exposure period. Because of the relatively high concentrations and the polar nature of the compound, the shape of the chromatographic peak showed tailing. However, because of the high concentrations, the peak shape did not detract from instrument precision or accuracy. Instrument precision was better than 10% based on multiple injections of calibration solutions.

Measurement of BD-diol in Plasma and Urine

The method for the analysis of BD-diol in plasma or urine was developed based on a butylboranate derivatization procedure described by Giachetti and coworkers (1989). In brief, plasma or urine was deproteinized by acidification with acetic acid followed by centrifugation in a tabletop centrifuge. Supernatants were derivatized, extracted into dichloromethane (Fisher Scientific), and centrifuged again. The extract was then analyzed by GC/MS as described below.

Human plasma and urine were used to develop methods for measuring BD-diol because these substrates were readily available and had been approved by the Institutional Review Board for use in an existing protocol. Use of human plasma and urine allowed methods to be developed, verified, and executed without killing animals for plasma and urine. During method verification, parallel studies comparing the recovery and linearity of BD-diol were conducted using human and rodent samples. The data showed no significant difference (within 10%) between the measurements obtained in human and rodent matrices.

Reagents and Standards Three solutions were created for the extraction: (1) an ammonium hydroxide mixture of 65 µL of 25% ammonium hydroxide with 10 mL of acetone; (2) a 0.2% acetic acid solution consisting of 20 µL acetic acid diluted into 10 mL of acetone; and (3) a butaneboronic acid solution consisting of 100 mg butaneboronic acid, 10 mL 2,2-dimethoxypropane, and 60 µL of 6 M HCl. Water was purified in-house by distillation of deionized water. Analytical-grade dichloromethane and acetone were purchased from Fisher Scientific; and butaneboronic acid, ammonium hydroxide (25%), dimethoxypropane, HCl, and acetic acid were purchased from Aldrich. Nitrogen gas was industrial grade.

Aliquots of BD-diol purchased for animal exposures were used for creating stock standards. Standards were preweighed gravimetrically and constituted in human plasma or nonexposed urine. Standards were stored at

−80°C when not in use. Calibration standards were made at the time of analysis by serial dilution of stock standards.

Sample Preparation Plasma or urine was thawed at room temperature until there was no ice in the sample. An aliquot (0.3 mL plasma or urine for rats; or 0.1 mL plasma or urine + 0.2 mL of water for mice) was transferred to a 500-µL microcentrifuge tube and acidified with a 0.2% solution of acetic acid to deproteinize it. The deproteinized sample was vortexed for 10 seconds and centrifuged at 4500 *g* for 3 minutes in a tabletop centrifuge. The resulting supernatant was transferred to a 15-mL polypropylene centrifuge tube and combined with 0.25 mL of the butaneboronic solution described above. This mixture was shaken by hand for approximately 5 seconds and allowed to react at room temperature for 20 minutes. The reaction was quenched with the addition of 1 mL methylene chloride and placed on a rotating shaker for 5 minutes. The resulting extract was centrifuged at 840 *g* for 4 minutes, and 1 mL of the lower portion of the supernatant (dichloromethane-containing analyte) was transferred to a 2-mL vial for analysis.

Instrumentation and Chemical Analysis Splitless injections (1 µL) were placed into a GC/MS (model 5890 GC; model 5972 MS; Hewlett Packard) equipped with a phenylmethylsilicone fused-silica capillary column (30-mm, 0.25 mm × 0.25 mm; RTX-5MS, Restek). The mass spectrometer was operated in the selective-ion-monitoring mode with default tune parameters after performing a maximum-sensitivity autotune. Operating conditions were as follows: 42°C for 0.6 minutes, heat 20°C/minute to 150°C, then heat 25°C/minute to 225°C. BD-diol was analyzed by selective ion monitoring of ion 139 for quantitation. Two qualifier ions, 154 and 68, were also monitored to verify the identity of the compound. The limit of detection for quantitation of BD-diol was assigned as the lowest concentration that could be sequentially analyzed ($n = 6$) and meet a predetermined precision acceptance criterion (coefficient of variation < 20%). The limit of quantitation was approximately 40 ng/mL, based on replicate precision and accuracy. At 40 ng/mL, the replicate precision ($n = 6$) and accuracy were within 20% of nominal values. At this concentration, the signal-to-noise ratio was greater than 10. BD-diol could be detected at lower concentrations (the lowest limit of detection was not formally determined), but the accuracy and precision were not acceptable for purposes of reporting quantitative values. Each set of biologic sample analyses consisted of at least 10% sample replicates and laboratory blanks of the extraction solvents.

Absolute recoveries of BD-diol in plasma and urine were not determined. All calibration curves were created by spiking BD-diol into the actual matrix of interest (plasma or urine) and the recovery of the extracted analytical samples was high enough to quantify BD-diol precisely and accurately at the set limit of quantitation. The extraction and recovery was linear throughout the entire calibration curve and was reproducible within 10% from day to day (new calibrations were conducted with every batch of samples). The stability of the BD-diol derivatives was determined by reanalysis of derivatized samples more than 24 hours after initial analysis; no degradation was observed. Stability of underivatized BD-diol in plasma and urine was not determined.

Measurement of M1 and M2 in Urine

The method for the analysis of M1 and M2 in urine was based on isolating these metabolites by solid-phase extraction and measurement using liquid chromatography and triple-quadrupole mass spectrometry. A more detailed description of the procedure and its optimization using, in part, biologic samples from this project, is published elsewhere (McDonald et al. 2004).

Reagents and Standards Analytical-grade ethyl acetate and methanol were purchased (Fisher Scientific). Water was purified in-house by distillation of deionized water. Potassium chloride, sodium chloride, urea, and HCl (Aldrich) were the highest grades available. Nitrogen gas was industrial grade. The M1 standard was acquired from Peter J. Boogaard (Shell Laboratories, Amsterdam, The Netherlands) and was determined to be more than 95% pure by HPLC (with a UV detector) and NMR. The M2 standard was synthesized in-house using the procedure described by Sabourin and associates (1992) and was determined to be more than 95% pure by HPLC (with a UV detector).

Preparation of Synthetic Urine, Calibration Standards, and Internal Standards Synthetic urine was created to establish calibration standards. Real human or rodent urine was not used to create standards because neither is "M1-free," as evidenced by the presence of endogenous M1 in control samples (Albertini et al. 2001, 2003). M2 has also been observed in some urine from rodent and human control subjects, as described later in this report. Synthetic urine was created to represent normal human physiological concentrations of salt and pH as reported by Tietz (1986). The urine contained 0.128 M NaCl, 0.06 M KCl, 0.05 M NaH_2PO_4, and 0.18 M urea, at pH 5.5 to 6.

M1 and M2 standards were preweighed gravimetrically and constituted in purified water. Calibration standards were made by serial dilution of stock standards with synthetic urine. The calibration spanned from the limit of

quantitation to approximately 1000 ng/mL. Stock standards were stored at −80°C when not in use.

Sample Preparation Urine was thawed at room temperature until there was no ice in the sample. Aliquots (1-mL) were removed and placed in 1.5-mL microcentrifuge tubes. The tubes were then centrifuged at 700 *g* for 2 minutes. Aliquots (0.5-mL) of the centrifuged-sample supernatant and 0.5 mL of purified water were placed in a duplicate set of microcentrifuge tubes and mixed well. This mixture was then passed through C_{18} solid-phase extraction cartridges (model WAT03815; Waters Corp., Milford, MA) prewetted with 2 mL methanol. Each column was first conditioned with 2 mL purified water. The flow rate through the columns was maintained at about 1 drop/second. Positive pressure from a pipette bulb or compressed air source was used to maintain the flow rate, if necessary, but at no time was the flow rate greater than approximately 1 drop/second. The column was not allowed to dry out between steps. The analytes were eluted from the column with 2 mL water into a labeled 2.0-mL collection tube, and a 1.0-mL aliquot was transferred to a vial for analysis.

Instrumentation and Chemical Analysis For the tandem mass spectrometry analysis of M1 and M2, liquid chromatography separation was conducted using a microplunger pump (model LC-10AD*VP*; Shimadzu Oceania, Sydney, Australia) fitted with an HPLC column (3 µm, 125 Å, 75 × 200 mm; model C_{18} Aqua, #003-4311-B0; Phenomenex, Torrance, CA). The column temperature was set at 32°C using a temperature-control modulator (model TC-50; Eppendorf, Westbury, NY). The flow rate was 350 µL/min. The injection volume was 25 µL with 100 µL over-fill (autoinjector). The solvent program is shown in Table II of McDonald and colleagues (2004). Under these conditions the M1 and M2 retention times were approximately 3.0 and 3.3 minutes, respectively.

Triple-quadrupole mass spectrometry (model API 365 LC/MS/MS; Applied Biosystems, Foster City, CA) and data analysis (version 1.3.1, Sciex Analyst; Agilent Technologies, Palo Alto, CA) were performed. Analytes were ionized using a heated nebulizer ionization source. The instrument was run in multiple-response-monitor mode and used the parent→daughter ion pairs of 250→121 and 232→103 atomic mass units to identify and quantify M1 and M2. The limits of quantitation for M1 and M2 were determined as described above for BD-diol and as reported in detail by McDonald and colleagues (2004). The limits of quantitation for M1 and M2 were assessed as the lowest concentrations that could be sequentially analyzed ($n = 6$) and meet a predetermined precision acceptance criterion (coefficient of variation < 20%). The limits of quantitation were 4.1 ng/mL for M1 and 1.4 ng/mL for M2. Each analysis consisted of at least 10% sample replicates and laboratory blanks of the extraction solvents.

Inhalation Study Protocols

Inhalation Study A. Nose-Only Exposure to BD (at Levels Used in Earlier Studies) for Measurement of BD-diol in Plasma Male and female mice ($n = 6$/sex/exposure level) and rats ($n = 5$/sex/exposure level) were exposed nose-only to BD (0, 62.5, 625, or 1250 ppm) for 6 hours. The primary goal was to determine the differences in resulting plasma concentrations of BD-diol that may be related to sex, species, and exposure level. Secondary goals were to (1) evaluate the sensitivity of the analytical method adapted for measuring BD-diol, and (2) collect additional tissues for future studies of sex- and species-related formation of BD-diol and epoxy metabolites of BD.

Animals were first conditioned to being placed in restraint tubes for a 6-hour period for 2 consecutive days. Individual animals were then placed in the exposure system at 3-minute intervals and, at the end of the exposure period, removed at the same rate for immediate necropsy. Each animal was injected with pentobarbital solution, the thorax was opened, whole blood was collected into heparinized syringes/tubes by cardiac puncture, and then additional tissues (heart, liver, lung, ovaries, and thymus) were harvested and snap-frozen for storage at −80°C. Blood samples were mixed with heparin, transferred to 2.0-mL Eppendorf tubes, and centrifuged in a refrigerated microfuge to separate plasma from cells. Aliquots of plasma from each animal were transferred into prelabeled 500-µL Eppendorf tubes, snap frozen, and stored at −80°C until analysis for BD-diol as described.

Inhalation Study B. Nose-Only Exposure to Low Levels of BD-diol for Measurement of BD-diol in Plasma Male and female mice and rats (n = 6/sex and species/exposure level) were exposed nose-only to BD-diol vapor (0, 6, or 18 ppm) for 6 hours. The primary goals were to (1) determine the differences in resulting plasma concentrations of BD-diol related to sex, species, and exposure level; and (2) compare these data with those obtained in animals exposed to BD (in Inhalation Study A) to estimate which BD-diol exposure levels would produce the same BD-diol plasma concentrations as those resulting from exposures to 62.5 and 625 ppm BD.

Animals exposed to BD-diol were conditioned, exposed, and necropsied for collection of plasma and tissues as described for Inhalation Study A.

Inhalation Study C. Whole-Body Exposure to Low Levels of BD-diol for Measurement of* Hprt *Mfs Female and male mice and rats were exposed in whole-body inhalation chambers to BD-diol vapor (0, 6, or 18 ppm) for 4 weeks (6 hours/day, 5 days/week). The chief purpose was to determine the mutagenic responses of *Hprt* M*f*s in splenic T cells after repeated exposures directly to BD-diol (rather than indirectly via exposure to BD). A secondary goal was to measure plasma concentrations of BD-diol that may be related to sex or species.

Immediately after the last day of exposure, groups of mice and rats (n = 6/sex and species/exposure level) were necropsied to collect blood and tissues for analysis of BD-diol. The remaining groups of female exposed and control animals were necropsied at 2, 4, 5.5, 7, or 8 weeks after exposure (n = 5/species/exposure level/time point) for isolation of splenic T cells and measurement of *Hprt* M*f*s. Groups of male mice and rats were necropsied only at 4 or 5 weeks after exposure (n = 5/species/exposure level/time point) to determine if the exposure was mutagenic and, if so, to collect *Hprt* mutant T-cell colonies for future molecular analyses.

The mutagenic responses in female mice and rats exposed to BD-diol in this Inhalation Study were compared with *Hprt* M*f* data from female animals exposed for 4 weeks to a range of BD exposures in earlier studies (Walker and Meng 2000).

Inhalation Study D. Nose-Only Exposure to BD (an Intermediate Level) for Measurement of BD-diol in Plasma and Urine Based on the plasma concentrations of BD-diol found in female mice and rats exposed nose-only to BD (in Inhalation Study A) or BD-diol (in Inhalation Study B) we conducted a follow-up study with an intermediate (200 ppm) BD exposure level. Female mice and rats (n = 6/species/exposure level/necropsy time point) were exposed nose-only to 0 or 200 ppm BD for 6 hours. Primary goals were to (1) determine plasma concentrations of BD-diol immediately after exposure (and to compare them with those found after exposure to 6 or 18 ppm BD-diol [in Inhalation Study B]); (2) determine the half-life of BD-diol in plasma; (3) determine whether BD-diol was unmodified when excreted in urine; and (4) compare concentrations of M1 and M2 in urine with concentrations of BD-diol in plasma and urine.

To assess the half-life value of BD-diol in plasma, female mice and rats were necropsied at 0, 2, 4, 6, or 24 hours after exposure (n = 6/species/exposure level/time point). The animals to be necropsied at 24 hours were placed in metabolic cages immediately after BD exposure to collect 24-hour urine samples over ice.

Inhalation Study E. Nose-Only Exposure to High Levels of BD-diol for Measurement of BD-diol in Plasma

Based upon the plasma concentrations of BD-diol found in female mice exposed nose-only to 6 or 18 ppm BD-diol (in Inhalation Study B), we conducted a follow-up study using higher BD-diol exposure levels. Female mice (n = 6/exposure level) were exposed nose-only to 0, 24, or 36 ppm BD-diol for 6 hours.

All animals were necropsied immediately after exposure to measure plasma levels of BD-diol and to compare the resulting data with those obtained in Inhalation Studies A, B, and D.

Inhalation Study F. Whole-Body Exposure to a High Level of BD-diol for Measurement of* Hprt *Mfs Based on the plasma concentrations of BD-diol and the mutagenic responses after exposure to BD-diol (Inhalation Studies B, C, and E) or to BD (Inhalation Studies A and D), we conducted a follow-up study at the highest level of BD-diol for a longer exposure duration to measure Hprt Mfs. (To decide the exposure level, we compared the plasma levels of BD-diol after the 6-hour exposures to 18, 24, and 36 ppm BD-diol in Inhalation Studies B, C, and E [see the Results section].) Female mice and rats and male mice (but not male rats; n = 5–8/sex and species/exposure level/necropsy time point) were exposed to 0 or 36 ppm BD-diol for 4 weeks (6 hours/day, 5 days/week). The primary goal was to measure Hprt Mfs after exposure to this higher level of BD-diol and to compare the resulting data with those found in Inhalation Study C (0, 6, or 18 ppm BD-diol, 4 weeks) and those previously reported from earlier studies with mice and rats exposed to BD (Walker and Meng 2000).

Groups of animals were necropsied at 2, 4, 5, 6, or 8 weeks after exposure for isolation of splenic T-cells and measurement of *Hprt* M*f*s. *Hprt* mutant clones from male mice were stored for future molecular analyses.

SPECIFIC AIM 5. BD MUTATION-SPECTRA EXPERIMENTS

RT–PCR Analysis of *Hprt* Mutant Clones from Mice and Rats

Preparation of Total RNA Frozen pellets of *Hprt* mutant clones that had been expanded as described previously (Meng et al. 1998b, 2004; Walker and Meng 2000) were thawed on ice and 1 to 10×10^3 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-Aldrich). 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.

RT–PCR The RT–PCR method for mouse *Hprt* was the same as previously reported (Meng et al. 1999a). The method for rat *Hprt* (as described by Chen et al. 1999) was slightly modified with changes in PCR buffer. For the initial RT–PCR amplification of *Hprt* mRNA, 4 µL cell lysate was used in a final volume of 20 µL RT–PCR mixture containing 2 µL 10 × VM buffer (10 mM $MgCl_2$; Promega; Walker and Skopek 1993), 0.4 µL deoxyribonucleoside triphosphate (dNTP; 25 mM; Promega), 0.4 µL oligo dT (0.5 µg/µL; Applied Biosystems), 0.4 µL AMV reverse transcriptase (5 U/µL; Promega), 0.4 µL *Tfl* DNA polymerase (5 U/µL; Promega), 10.4 µL sterile double-distilled H_2O, and 1.0 µL of either mouse or rat *Hprt*-specific 5′ primer (10 µM, M101F: 5′-TTA CCT CAC TGC TTT CCG GA-3′ for mouse *Hprt* or 10 µM, R1F: 5′-CTT CCT CCT CAG ACC GCT TT-3′ for rat *Hprt*) and 3′ primer (10 µM, M901R: 5′-GAT GGC CAC AGG ACT AGA AC-3′ for mouse *Hprt* or 10 µM, R1R: 5′-TGG CCA CAG GAC TAG AAC GT-3′ for rat *Hprt*).

The PCR program was conducted for 45 minutes at 48°C and then 2 minutes at 94°C, followed by 40 cycles of 30-second denaturation at 94°C, 1 minute of annealing at 55°C, and 2 minutes of extension at 68°C. The final cycle included an additional 7-minute extension at 68°C. The product of this reaction was diluted 1:100 in sterile H_2O, and 1 µL of the dilution was used as the cDNA template in a nested PCR. The 30-µL nested PCR reaction mixture contained 3 µL 10 × VM buffer (27.5 mM $MgCl_2$), 0.3 µL AmpliTaq DNA polymerase (5 U/µL; Applied Biosystems), 22.7 µL sterile double-distilled H_2O, and 1 µL of either mouse or rat *Hprt*-specific 5′ primer (10 µM, M102F: 5′-GGC TTC CTC CTC AGA CCG CT-3′ for mouse *Hprt* or 10 µM, R2F: 5′-GCG AGC CGA CCG GTT CTG TC-3′ for rat *Hprt*) and 3′ primer (10 µM, M902R: 5′-GGC AAC ATC AAC AGG ACT CC-3′ for mouse *Hprt* or 10 µM, R2R: 5′-TTC TTT ACT GGC CAC ATC AA-3′ for rat *Hprt*). The reaction mixture was heated for 4 minutes at 94°C, followed by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. The final cycle included an additional 7-minute extension at 72°C. A 5-µL aliquot of the nested PCR product was run on an 8% polyacrylamide gel to assess PCR efficiency.

Concomitant with this work, another investigator in our laboratory found a slightly different protocol for RT–PCR of mouse *Hprt* that appeared to yield more product. This protocol involved using 3.6 mM $MgCl_2$ per reaction vial (rather than 1 mM $MgCl_2$) in the RT–PCR reaction and annealing at 51 to 52°C (rather than 55°C). For the nested PCR reaction, 5.8 to 6.0 mM $MgCl_2$ per reaction vial was found to be optimal, with annealing at 52°C. This modified protocol was used thereafter for RT–PCR of mouse RNA isolated from *Hprt* mutant clones analyzed in this study.

For direct sequencing of *Hprt* PCR products, the remainder of the nested PCR products was filtered using concentrators (model Centricon 50, Amicon, Beverly, MA), and the filtrates were vacuum-dried and resuspended in 20 µL sterile water as templates for single-strand PCR in both directions, using the appropriate 5′ primer or 3′ primer for mouse *Hprt* (M102F or M902R primers, respectively) or for rat *Hprt (*R2F or R2R primers, respectively). Single-strand PCR products were filtered through spin columns (Centri-Sep, Princeton Separations, Adelphia, NJ), and aliquots of these PCR products were then sequenced using the Sequenase/dideoxy protocol specified by Applied Biosystems.

Multiplex PCR Analysis of *Hprt* Mutant Clones from Mice and Rats

Genomic DNA Isolation Genomic DNA was extracted from expanded *Hprt* mutant clones using a DNA isolation kit (PUREGENE, Gentra, Minneapolis, MN) and following the manufacturer's protocol for cultured cells.

Multiplex PCR All nine *Hprt* exons were amplified simultaneously from either mouse or rat genomic DNA, resulting in a single PCR product for exons 7 and 8 and individual PCR fragments for each of the other seven exons for each species. Information about the development and use of primers for mouse *Hprt* is given in the Results section. The sequences for the oligodeoxynucleotide primers and the PCR conditions for the rat *Hprt* were described previously by Chen and coworkers (1999). In addition, the unlinked exon 2 of the K-*ras* gene was amplified in the same reaction as an internal positive control, using the same primers for both mouse and rat genomic DNA (Chen et al. 1999). The amplification reactions for multiplex PCR of mouse or rat *Hprt* were carried out in a total volume of 30 µL in 500-µL microcentrifuge tubes. Each reaction mixture consisted of 0.5 µg genomic DNA, 5 U AmpliTaq DNA polymerase, 125 mM KCl, 25 mM Tris-HCl (pH 8.3), 3.75 mM $MgCl_2$, 250 µM of each of four dNTPs, 5% DMSO, and the nine pairs of primers (including, for each species, the eight pairs of primers for the nine *Hprt* exons and a primer pair for exon 2 of the K-*ras* gene). The reaction was incubated for 5 minutes at 94°C, followed by 33 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. The final cycle included an additional 7-minute incubation at 72°C. The reaction products (10 µL) were separated by electrophoresis through a 2% agarose gel that contained 0.5 µg/mL ethidium bromide and were viewed on a UV transilluminator.

STATISTICAL ANALYSES

Sample-Size Estimates for Mutagenicity Studies

The primary endpoint investigated in this project was *Hprt* M*f*s in the splenic T cells of chemically exposed rodents. Yet this proved to be the least sensitive endpoint compared with sham-exposed control animals. In our earlier HEI-supported exposure–response studies of mutagenicity in BD-exposed mice and rats (Walker and Meng 2000), female mice necropsied 2 weeks after a 2-week exposure to 20 ppm BD (the time of peak mutagenic response in splenic T cells for the age of animal and exposure regimen used) showed a highly significant twofold increase ($P < 0.001$) in the mean *Hprt* M*f* ($n = 9$, M*f* = 3.4 ± 0.8 × 10^{-6}) over that of control mice ($n = 5$, M*f* = 1.7 ± 0.2 × 10^{-6}; Meng et al. 1999b). These data were used to perform power calculations, which estimated that 20 or 25 mice per group would be required to result in a 70% or 80% chance, respectively, of detecting a significant mutagenic effect (approximately 1.5-fold over background *Hprt* M*f*s) in female mice after a 2-week exposure to 3 ppm BD. This low-level BD-exposure regimen (used for Specific Aim 1) was expected to have substantially less mutagenic potency than the other exposure regimens (for BD, BDO_2, and BD-diol) planned for the mutagenicity studies in this project. For this reason, we used 27 exposed and 24 control female mice for Specific Aim 1 and typically 4 to 6 animals for each exposure and control group for Specific Aims 2 through 4 to detect mutagenic effects greater than twofold.

Hypothesis Testing: Analysis of CE and *Hprt* M*f* Data (Specific Aims 1 Through 4)

At specific time points after exposure to BD or one of its metabolites, the Mann-Whitney Rank Sum test was used to describe the differences in CE or M*f* values between groups of control and exposed animals (Hollander and Wolfe 1973). When the pair-wise (exposed and control) comparisons for more than one time point were significant in an experiment that examined the change in *Hprt* M*f* over time, Bonferroni adjustments of the *P* values were made. Correlation coefficients between the natural log of the *Hprt* M*f* and the natural log of the CE for control and BD-exposed mice were computed as the Pearson product-moment correlation coefficient (Hollander and Wolfe 1973). Best-fit regression curves for the CE and *Hprt* M*f* data for control and BD-exposed mice were derived from a sigmoidal equation fitting the five best parameters (SigmaPlot; SPSS, Chicago, IL). *P* values obtained were considered significant when ≤ 0.05.

Estimation of Mutagenic Potency (Specific Aims 2 Through 4)

Although many articles have been published on analyses performed in potency experiments, most are confined to considering parallel line assays, in which responses to different exposure levels are observed at a single point in time. The current investigation, as well as our earlier HEI project (Walker and Meng 2000), involved an assay in which nonlinear responses to a single exposure level were observed over time. A literature search failed to identify an existing technique for estimating mutagenic potency in temporal data of this type. We therefore adapted the technique of summarizing the response in terms of total mutagenicity by fitting and integrating a polynomial model with respect to time. The resulting estimate of mutagenic potency is a relative value that represents the number of mutant cells passing through the spleen over the period of time being evaluated. It approximates the following: (number of *Hprt* mutants/number of wild-type cells) × (number of wild-type cells passing through the spleen/unit of time).

The method we developed for estimating the mutagenic potency of any given chemical exposure regimen in splenic T cells from exposed versus control rodents is described in detail elsewhere (Meng et al. 1998a, 1999a; Walker and Meng 2000); in brief, it employs the following steps:

1. The best-fit predicted value at each time point is used as the starting value to which normally distributed random variates on a log scale with (zero mean, robust regression-estimated residual variance) are added (the number of variates corresponding to the observed sample size).
2. A cubic curve is fitted with robust regression to this simulation of the dataset.
3. A potency estimate is obtained by integrating over the back-transformed cubic curve.
4. Steps 1 to 3 are repeated over 5000 simulations.
5. The 2.5th and 97.5th percentiles of the potency estimates are taken as the 95% confidence limits.

The confidence intervals (CIs) for our earlier mutant-manifestation studies of BD and racemic mixtures of BDO_2 and BDO were constructed using a simulation based on estimated mean and variance (Meng et al. 1998a, 1999a,b; Walker and Meng 2000). However, resampling (e.g., bootstrapping) is a viable option to estimate the value of the integrated-response potency statistic and its associated distribution. Whereas resampling makes no assumptions about the distributional features of the data distribution (e.g., mathematical form, shape) and circumvents the issue

of adequately addressing uncertainty in estimating the variance in M*f* at each time point for *Hprt* mutant T-cell manifestation curves, bootstrapping amounts to replacing the data simulation in step 1 above with another type of "simulation" (i.e., constructing many copies of the observed data and resampling them). To test the numerical equivalence of these two approaches, we ran a bootstrap (resampling) algorithm to obtain mutagenic potency CIs for data sets from our earlier studies of the mutagenicity of BD in mice and rats (Meng et al. 1998a, 1999a). The bootstrap intervals proved to be slightly longer than those produced by our approach, but the difference was not substantial enough to have an appreciable effect on statistical inference. (An example of the CIs obtained by both approaches for one data set [i.e., *Hprt* M*f*s in splenic T cells from 4- to 5-week-old female mice exposed for 2 weeks to 1250 ppm BD] is provided in Table 4.) Given the conceptual and demonstrated comparability to the approach we used previously, the same method was applied in the generation of CIs for the estimates of mutagenic potency for rodents exposed to BD, *meso*-BDO_2, or BD-diol in the current project.

Hypothesis Testing: Analysis of *Hprt* Mutation-Spectra Data (Specific Aim 5)

Several approaches were used to evaluate the effects of BD exposure on mutation spectra, proportions of individual types of mutations, and mutant fractions in rats and mice. The statistical significance of differences in mutation spectra, in terms of the proportional distribution of mutations of different types in exposed and control rats, was first assessed using the generalization of the Fisher exact test to R × C contingency tables (Freeman and Halton 1951). The Fisher exact test for 2 × 2 contingency tables was used to evaluate the significance of differences in proportions of individual types of mutations in exposed and control groups. Statistical testing was two-sided, with significance assessed at $P = 0.05$.

Mutant fractions (calculated as [average observed M*f*] × [average percent of total mutants for a type of mutation]) for individual mutation types were analyzed under the assumption that mutation counts were distributed as Poisson variants. The conditional distribution method for comparing Poisson means (Lehmann 1959) was used to compare mutant fractions in control and BD-exposed animals. The null hypothesis was expressed as: The probability of a mutation event occurring in a BD-exposed animal was not significantly different from the probability of the same event occurring in a control animal. Statistical testing was one-sided against the alternative that the mean mutant fraction in BD-exposed animals exceeded that of controls. *P* values for these comparisons should not be overinterpreted. The tests do not take into account interanimal variability in Poisson means, which if substantial would create an overdispersed Poisson distribution and underestimated *P* values, leading to a larger probability of false-positive inferences than would be expected under the hypothesis tested at the nominal level ($P = 0.05$).

RESULTS

SPECIFIC AIM 1. LOW-LEVEL BD EXPERIMENTS

Actual exposure concentrations for BD were determined using periodic samplings of the inhalation chamber atmospheres. The average chamber concentration for the low-level exposure group (female mice 4 to 5 weeks old) was 3.06 ± 1.76 ppm for the 10 exposure days (6 hours/day, 5 days/week).

Effects of Exposure on CEs and *Hprt* M*f*s in Splenic T Cells of Female Mice Exposed to Low-Level BD

Cloning Efficiency Surprisingly, there was a significant difference in the growth of splenic T cells from control mice ($n = 24$) and mice exposed to 3 ppm BD ($n = 27$; Table 2, $P = 0.004$). The average T-cell CEs were 9.63% (range of 5.77 to 15.63%) for control and 7.69% (range of 4.68 to 15.63%) for exposed mice. This finding was unexpected because in earlier mutagenicity studies significant reductions in T-cell growth were not seen with female mice of similar ages exposed for up to 4 weeks to a wide range of BD levels (20 to 1250 ppm; Meng et al. 1998a, 1999a; Walker and Meng 2000). These data alone, from female mice exposed to 3 to 1250 ppm BD, might suggest that DNA repair is induced at higher BD levels and not induced at lower levels. However, the average CE in mice exposed to nominal 1750 ppm BD (5.79%, range of 4.40 to 7.31%, $n = 4$) was also significantly lower by 40% of the average value for unexposed animals ($P = 0.001$; see Meng et al. 2001). Furthermore, significantly lower CEs have been observed previously in female mice exposed for 4 weeks to 2 or 4 ppm (±)-BDO_2 (Meng et al. 1999b; Walker and Meng 2000). In those studies exposures to 3.6 ppm BDO_2 and 62.5 ppm BD produced comparable blood concentrations of BDO_2. Comparing these earlier data with those from this study suggests that BD might be more cytotoxic to mouse T cells than heretofore recognized and that the ability to detect a significant decrease in CE in mice exposed to 3 ppm BD in this study was enhanced by using a sample size much larger than usual for both the control ($n = 24$) and exposure groups ($n = 27$).

Table 2. CE and *Hprt* M*f* Data for Individual Female Mice Exposed to 0 or 3 ppm BD (Specific Aim 1)[a]

Animal Number	Positive Wells / Total Wells in Non-Selection Plates	CE (%)	Positive Wells / Total Wells in 6-TG Plates	M*f* per Clonable Cell ($\times 10^{-6}$)
Control group				
A110	92/192	8.15	1/384	0.80
A111	124/192	12.97	1/732	0.26
A112	106/192	10.04	2/456	1.09
A113	111/185	11.45	1/420	0.52
A114	118/192	11.92	1/456	0.46
A115	102/192	9.47	2/564	0.94
A116	86/192	7.43	2/576	1.17
A117	102/192	9.47	2/516	1.03
A118	132/192	14.54	1/528	0.17
A119	71/192	5.77	1/324	1.34
A120	106/192	10.04	4/660	1.52
A121	77/192	6.41	1/480	0.33
A122	119/192	12.09	1/396	0.53
A123	137/192	15.63	1/384	0.42
A124	115/192	11.42	2/420	1.04
A125	85/189	7.47	1/384	0.87
A126	81/192	6.85	1/456	0.80
A127	79/187	6.86	2/324	2.26
A128	110/191	10.70	1/300	0.78
A129	96/192	8.66	1/372	0.78
A130	99/192	9.06	3/708	1.17
A131	90/192	7.91	2/480	1.32
A132	104/192	9.75	3/720	1.47
A133	83/192	7.08	3/564	1.88
Average ± SD		9.63 ± 2.59		0.96 ± 0.51

Table continues next page

[a] Groups of control and exposed female mice (4 to 5 weeks old) were necropsied at 4 weeks after 2 weeks of exposure (6 hours/day, 5 days/week). Splenic T cells were isolated, primed with concanavalin A, and cultured with rat T-STIM and IL-2. CEs and M*f*s were measured at 8 to 9 days after plating.

[b] $P = 0.004$, Mann-Whitney Rank Sum test of the difference between control and BD-exposed mice.

Hprt Mfs To extend earlier exposure–response data for *Hprt* M*f*s in splenic T cells of female B6C3F1 mice exposed to 20 to 1250 ppm BD (Meng et al. 1999a; Walker and Meng 2000), we exposed female mice for 2 weeks to 3 ppm BD as a new low-exposure level. The average *Hprt* M*f* in mice exposed to 3 ppm BD was $1.54 \pm 0.82 \times 10^{-6}$ ($n = 27$), which was a significant increase of 1.6-fold over the average M*f* for control mice of $0.96 \pm 0.51 \times 10^{-6}$ ($n = 24$, $P = 0.004$; Figure 4).

The Mf–CE Relation Compared with Earlier Mouse and Human Studies Because low CEs of peripheral blood T cells have been shown to affect the frequency of background *Hprt* mutations in some population studies of adult humans (Albertini 1985; Cole et al. 1988; Tates et al. 1991), analyses of the relation between CE and M*f* in control and BD-exposed mice were carried out in the current study. These analyses were possible because the relatively large numbers of mice used in the study substantially exceeded the typical sample sizes of 5 to 10 per group of unexposed and exposed mice in previous studies (Cochrane and Skopek 1994b; Tates et al. 1994; Meng et al. 1998a, 1999a).

Correlation coefficients between the natural log of the M*f* and the CE for the control mice ($n = 24$) and those exposed to 3 ppm BD ($n = 27$) were calculated; a weak negative correlation was found for the control group ($r = -0.58$) but not

Table 2 (*Continued*). CE and *Hprt* M*f* Data for Individual Female Mice Exposed to 0 or 3 ppm BD (Specific Aim 1)[a]

Animal Number	Positive Wells / Total Wells in Non-Selection Plates	CE (%)	Positive Wells / Total Wells in 6-TG Plates	M*f* per Clonable Cell ($\times 10^{-6}$)
3 ppm BD group				
B021	68/192	5.47	1/480	0.95
B022	65/192	5.17	2/576	1.68
B023	108/192	10.33	2/324	1.50
B024	73/192	5.98	1/480	0.88
B025	106/187	10.46	4/576	1.67
B026	64/192	5.07	2/432	2.29
B027	73/192	5.98	1/480	0.87
B028	75/192	6.19	3/576	2.11
B029	78/192	6.52	1/480	0.80
B030	101/192	9.33	4/576	1.87
B031	78/192	6.52	1/480	0.80
B032	98/192	8.93	2/672	0.83
B033	66/190	5.33	2/564	0.89
B034	60/192	4.68	2/652	1.64
B035	70/192	5.67	2/624	1.42
B036	65/192	5.17	2/528	1.84
B037	76/192	6.30	2/525	1.51
B038	68/191	5.50	4/516	3.54
B039	137/192	15.63	4/648	0.99
B040	128/192	13.73	9/672	2.46
B041	84/192	7.19	2/576	1.21
B042	96/192	8.66	2/576	1.00
B043	98/192	8.93	1/576	0.49
B044	73/192	5.91	2/480	1.77
B045	104/192	9.75	3/480	1.61
B046	96/192	8.66	6/432	4.04
B047	110/192	10.63	2/480	0.98
Average ± SD		7.69 ± 2.77[b]		1.54 ± 0.82[b]

[a] Groups of control and exposed female mice (4 to 5 weeks old) were necropsied at 4 weeks after 2 weeks of exposure (6 hours/day, 5 days/week). Splenic T cells were isolated, primed with concanavalin A, and cultured with rat T-STIM and IL-2. CEs and M*f*s were measured at 8 to 9 days after plating.

[b] $P = 0.004$, Mann-Whitney Rank Sum test of the difference between control and BD-exposed mice.

for the exposed group ($r = -0.25$). A semi-log plot of the best-fit curve of the relation between M*f* and CE in control mice was basically a straight line with a slope of zero (i.e., a horizontal line) when CEs were at 6 to 11% (Figure 5) and seemed to slope downward somewhat at a CE higher than 11%. However, the significance of a possible weak effect of CE on M*f* remains in question given the fact that the deflection in the curve is driven largely by CE values for about 2 of 24 control mice (Figure 5). Also, among BD-exposed animals, there were two points in the CE range of about 14 to 16% and no downward deflection of the curve, as would be expected if a low CE had a significant inversely related impact on M*f*. Rather, the best-fit curve of the relation between M*f* and CE for BD-exposed mice was a straight horizontal line with a slope of zero (Figure 5). Although it appears that BD did decrease CEs a little, this effect pushes the values into a range where CE doesn't affect M*f*. These comparisons of CE and M*f*, based directly on statistical approaches applied in earlier *Hprt* mutagenicity studies in adult humans (Albertini 1985; Cole et al. 1988; Tates et al. 1991), apply a higher level of stringency to the evaluation of the *Hprt* M*f* data from control and BD-exposed mice. In the face of this higher stringency, low-level BD exposure at 3 ppm is still mutagenic in mice.

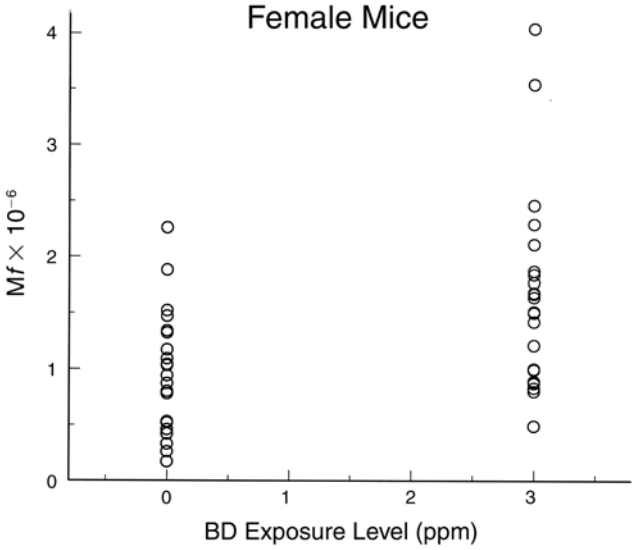

Figure 4. *Hprt* M*f*s in splenic T cells of female mice after inhalation exposure to low-level BD. Control (n = 24) and exposed (n = 27) animals were necropsied 4 weeks after cessation of a 2-week exposure to 0 or 3 ppm BD. *Hprt* M*f* values for individual control and BD-exposed mice are plotted. (Specific Aim 1)

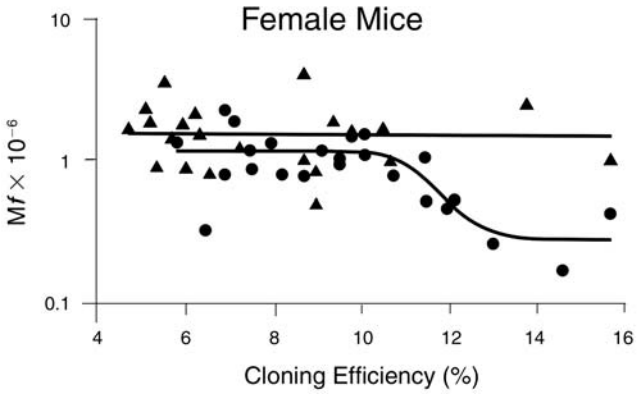

Figure 5. Best-fit regression curve for the relationships between *Hprt* M*f* and CE data for control and BD-exposed female mice. Control (n = 24) and exposed (n = 27) animals were necropsied 4 weeks after cessation of a 2-week exposure to 0 or 3 ppm BD. BD-exposed mice (▲, upper curve); control mice (●, lower curve). (Specific Aim 1)

We also assessed published CE and *Hprt* M*f* data from control mice and mice exposed to 20 ppm BD in a similar protocol (by inhalation 6 hours/day, 5 days/week, for 2 weeks; Meng et al. 1999a; Walker and Meng 2000) to determine whether CE had a different impact on M*f* in animals exposed to 20 ppm compared with those exposed to 3 ppm BD. Correlation coefficients between the natural log of the M*f* and the CE for the control mice (n = 5) and BD-exposed mice (n = 9) were calculated, and no significant relations were found in either group (control mice, $r = -0.31$; exposed mice, $r = 0.25$). These data suggest that the potential impact of low CE on *Hprt* M*f*s in mice is diminished compared with that in adult humans, where several interdependent covariates may affect M*f* values (Albertini 1985; Cole et al. 1988; Tates et al. 1991; see Discussion section).

Sample-Size Estimates for *Hprt* Mutagenicity Studies with Female Mice Exposed to Low-Level BD

Before we conducted the mutagenicity study of mice exposed to 3 ppm BD (Specific Aim 1), we performed power calculations using individual M*f* data for control mice and mice exposed to 20 ppm BD in an earlier study (Walker and Meng 2000) to estimate the population size we would need to provide an 80% chance of detecting a significant mutagenic response above background; it was determined to be 25 mice/group. In earlier studies (Walker and Meng 2000), we fitted an exposure–response curve to the M*f* data from female mice exposed to 20, 62.5, or 625 ppm BD. From that curve we were able to calculate that 2 weeks of exposure to 3 ppm BD should induce a 50% increase over average background (control) levels of *Hprt* mutations if the mutagenic response remained true to the plotted exposure–response curve. The mean experimental *Hprt* M*f* value for mice exposed to 3 ppm BD in this study was 54% greater than the mean value for control animals. The individual *Hprt* M*f* values for control mice and mice exposed to 3 ppm BD (Table 2) represent the most robust data set to date that demonstrates such a weak but significant mutagenic effect for chemically induced mutations in rodents.

The individual *Hprt* M*f* data in Table 2 were combined with those from female mice exposed to 20 or 62.5 ppm BD in an earlier study (Walker and Meng 2000) to produce a curve fitted to the average induced M*f*s across exposures from 3 to 62.5 ppm BD (Figure 6). The resulting curve provides a rare opportunity to project the number of animals required for detecting a statistically significant (P = 0.05) difference between control and test groups of female mice exposed to ≥ 1 ppm BD or to exposure regimens using other DNA-damaging agents that yield weak mutagenic responses. The estimated sample sizes shown in Table 3 are based on (1) an assumed control mouse mean M*f* of 1×10^{-6} and (2) the use of a simple t test; but these sample sizes should be reasonably reliable guidelines for other statistical tests. The power calculations shown are based on individual *Hprt* M*f* values for 24 control mice and 27 mice exposed for 2 weeks to 3 ppm BD. The power of detection is equal to the percentage of differences that would be statistically significant across the universe of samples of control and exposed animals. Thus, a 25% difference would imply an M*f* in the exposed group of 1.25×10^{-6} if the effect per unit dose were

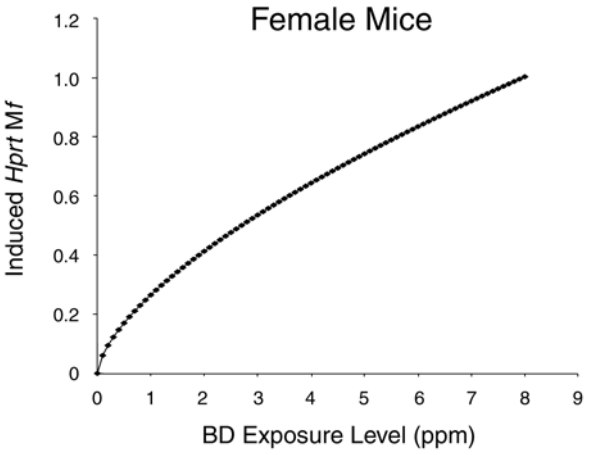

Figure 6. Detail of curve fitted to mean induced *Hprt* M*f*s in splenic T cells from female mice after a 2-week exposure to 3, 20, or 62.5 ppm BD. The curve includes interpolated values between actual BD exposure levels evaluated for mutagenicity. *Hprt* M*f* data for 3 ppm exposure are from this study (4 to 5 weeks old at start, necropsied 4 weeks after exposure; Table 2); data for 20- and 62.5-ppm exposures are from Walker and Meng (2000). (Specific Aim 1)

Table 3. Sample-Size Estimates from Power Calculations (Specific Aim 1)[a]

% Difference from Control[c]	Sample Size[b]		
	90% Power	80% Power	70% Power
10	605	437	333
25	111	81	62
40	50	36	28
50	35	26	20
60	26	19	15
75	19	17	11
100	13	10	8

[a] Power calculations (one-tailed $P = 0.05$) were based on individual *Hprt* M*f* data from control ($n = 24$) or exposed ($n = 27$) female mice. Groups of female mice (4 to 5 weeks old) were exposed for 2 weeks to 0 or 3 pm BD. They were necropsied at 4 weeks after exposure. *Hprt* M*f* data from Table 2 were used for power calculations.

[b] Estimated number of animals in each group (control and exposed).

[c] Approximate difference in observed M*f* from mice exposed to 0 or 3 ppm BD.

uniform across the range of BD exposures. It is important to note, however, that the exposure–response curve for BD-induced *Hprt* M*f*s is supralinear (i.e., with negative curvature); the effect per unit dose at 3 ppm BD, for example, is 2.3-fold greater than that at 20 ppm BD after exposure of the same duration in mice of the same age (Figure 6). Thus, the curve in Figure 6 projects that low-level exposures of female mice to 1.0 or 0.2 ppm, for example, should cause 27% or 10% increases, respectively, in background levels of *Hprt* M*f*s (Figure 6), and the 80% power sample-size estimates in Table 3 predict that detection of such small mutagenic effects at the $P = 0.05$ level would require about 80 mice per group (control and exposed) at 1 ppm BD and more than 400 animals per group at 0.2 ppm BD.

SPECIFIC AIM 2. AGE- AND SEX-RELATED BD EXPERIMENTS

Age- and Sex-Related Effects on T-Cell CEs and *Hprt* M*f*s in Control Animals and on T-Cell CEs in Exposed Animals

CEs The average inhalation chamber concentration for the 2-week exposure period (6 hours/day, 5 days/week) was 1253 ± 13 ppm BD. This exposure caused no clinical signs of toxicity or significant effects on CEs of T cells from exposed mice and rats (male mice and rats 4 to 5 weeks old or female mice 8 to 9 weeks old) compared with control animals. The average CEs in control or BD-exposed mice were 7.4% ($n = 36$) and 6.9% ($n = 52$), respectively. The average CEs in control and BD-exposed rats were 7.4% ($n = 5$) and 6.5% ($n = 15$), respectively.

***Mf*s** The average *Hprt* M*f* in 8- to 9-week-old control female mice was $2.2 \pm 0.9 \times 10^{-6}$ ($n = 25$), which was slightly higher than that in 4- to 5-week-old control female mice in our earlier study ($1.5 \pm 0.7 \times 10^{-6}$; Walker and Meng 2000). However, there was no significant difference between these and historical M*f* values (Skopek et al. 1992, 1996; Tates et al. 1994, 1998; Walker et al. 1996, 1997, 1998; Meng et al. 1998a,b, 1999a,b).

The average *Hprt* M*f* for 4- to 5-week-old control rats (male) was $4.2 \pm 1.9 \times 10^{-6}$ ($n = 5$), which is within the historical range of values of background *Hprt* M*f*s in F344 rats (Aidoo et al. 1991, 1993; Meng et al. 1998a, 1999a,b).

Also, there were no significant differences between sexes in average CE or *Hprt* M*f* values in control mice and control rats in this study or in our earlier BD-related mutagenicity studies (Walker and Meng 2000).

Age-Related Effects on *Hprt* M*f*s in Exposed Female Mice

To evaluate the effects of age on BD-induced mutagenic responses, 8- to 9-week-old female mice were exposed to 0 or 1250 ppm BD for 2 weeks (25 control and 22 exposed); *Hprt* M*f*s in splenic T cells were determined at 2, 5, 8, 11, and 13 weeks after exposure. The resulting M*f* data were compared with those obtained previously for 4- to 5-week-old female mice after similar BD exposures (Meng et al. 1998a; Walker

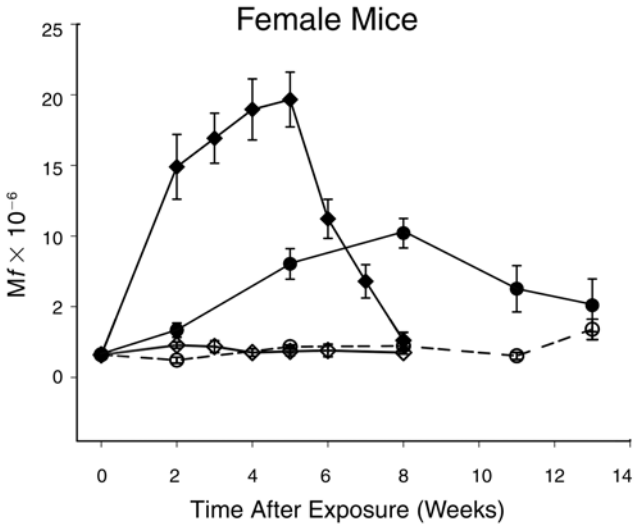

Figure 7. Effects of age and time elapsed after exposure on BD-induced mutagenic responses. A group of 8- to 9-week-old female mice were exposed for 2 weeks to 0 or 1250 ppm BD. *Hprt* M*f* data were determined at 2 to 13 weeks after exposure and are compared with data from an earlier study of similarly exposed 4- to 5-week-old female mice. Data points are means with SE bars. For 4- to 5-week-old mice, n = 4–10 exposed ◆ and controls ● per time point after exposure; for 8- to 9-week-old mice, n = 4–5 exposed ◇ and controls ○ per time point after exposure. *Hprt* M*f* data for 4- to 5-week-old mice are from an earlier study by Meng and associates (1998a). (Specific Aim 2)

and Meng 2000). In the 8- to 9-week-old mice, BD exposure caused significant increases in *Hprt* M*f*s at 2, 5, 8, and 11 weeks after exposure (P values from 0.001 to 0.028) but not at 13 weeks (P = 0.099; Figure 7). After Bonferroni adjustments were made on P values, the elevations in M*f*s in exposed mice remained at all time points except at 11 weeks, when M*f*s were returning to background levels. Maximum M*f*s were observed at 8 weeks after exposure in 8- to 9-week-old female mice ($10.2 \pm 2.1 \times 10^{-6}$) compared with 5 weeks after exposure in 4- to 5-week-old female mice exposed in a similar protocol in our earlier study (Figure 7; Meng et al. 1998a; Walker and Meng 2000).

To estimate the mutagenic potency of BD exposure at the *Hprt* locus in splenic T cells of 8- to 9-week-old female mice, curves were fitted to individual *Hprt* M*f* data for BD-exposed and control mice. The difference in the areas under the curves for exposed versus control mice represented the mutagenic potency under the given exposure regimen (Figure 7). The mutagenic potency of 2 weeks of inhalation exposure to 1250 ppm BD in 8- to 9-week-old mice was 57.0 (95% CI, 44.7–71.5). This was not significantly different from the estimated mutagenic potency of BD after similar exposures in 4- to 5-week-old mice (69.6; 95% CI, 61.1–78.7) in our earlier study (Table 4; Meng et al. 1998a; Walker and Meng 2000).

Table 4. Comparison of Methods to Calculate Mutagenic Potencies and CIs (Specific Aim 2)

Animal Age (Weeks)	Mutagenic Potency[a]	95% CI	
		Lower	Upper
4–5[b]	69.6	61.1[c]	78.7[c]
		59.9[d]	79.0[d]
8–9	57.0	44.7[c]	71.5[c]

[a] Estimates are based on *Hprt* M*f*s in splenic T cells from female mice of different ages after inhalation exposure to 0 or 1250 ppm BD.

[b] Mutagenic potency data for 4- to 5-week-old mice are from Meng et al. 1998a.

[c] CIs were constructed using distribution simulation—log-scale normality (Meng et al. 1998a, 1999a,b; Walker and Meng 2000).

[d] CIs were constructed using a bootstrapping approach.

Sex-Related Effects on *Hprt* M*f*s in Exposed Mice and Rats

Mice To compare sex-related differences in the mutagenic effects of exposure to 0 or 1250 ppm BD in mice, *Hprt* M*f*s were measured at the time of maximum mutagenic response after exposure, which was 5 weeks after a 2-week exposure of 4- to 5-week-old male mice. The resulting M*f* data were compared with those previously obtained after similar exposure of 4- to 5-week-old female mice (Meng et al. 1998a; Walker and Meng 2000). In the current study, the average *Hprt* M*f* in exposed male mice ($9.2 \pm 3.3 \times 10^{-6}$) was 6.2-fold greater ($P < 0.001$) than that in control male mice ($1.5 \pm 0.7 \times 10^{-6}$). In the earlier study with female mice (Meng et al. 1998a; Walker and Meng 2000), the *Hprt* M*f* attributable to BD exposure (the average M*f* in BD-exposed mice minus that in control mice) in female mice of similar ages (under the same exposure conditions) was 17.8×10^{-6}. This value was 2.3-fold greater than the *Hprt* M*f* attributable to BD exposure in male mice (7.7×10^{-6}) in the current study (Figure 8).

Rats To compare sex-related differences in the mutagenic effects of exposure to 0 or 1250 ppm BD in rats, *Hprt* M*f*s were measured at the time of peak mutagenic response after exposure, which is 4 weeks after a 2-week exposure of 4- to 5-week-old male rats. The resulting M*f* data were compared with those previously obtained after similar exposure of 4- to 5-week-old female rats (Meng et al. 1998a; Walker and Meng 2000). In the current study, the average *Hprt* M*f* in BD-exposed male rats ($8.1 \pm 2.8 \times 10^{-6}$) was 1.9-fold greater ($P$ = 0.01) than that in control male rats ($4.2 \pm 1.9 \times 10^{-6}$). In our earlier study (Meng et al. 1998a; Walker and Meng 2000), we showed that BD exposure

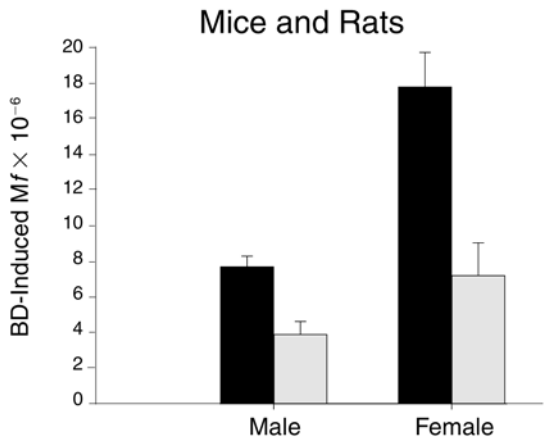

Figure 8. Sex- and species-related differences in BD-induced *Hprt* M*f*s in splenic T cells of mice and rats exposed to 1250 ppm BD for 2 weeks beginning at 4 to 5 weeks of age. Animals were necropsied at 4 or 5 weeks after exposure. Means of BD-induced M*f*s were obtained by subtracting the average M*f*s in control animals from those in BD-exposed animals. Mice (black bars); rats (gray bars); bars show average ± SD. M*f* data for females are from an earlier study (Meng et al. 1998a). (Specific Aim 2)

induced a mean *Hprt* M*f* of 7.2×10^{-6} (over background) in 4- to 5-week-old female rats. This value was 1.9-fold greater than *Hprt* M*f* attributable to BD exposure in male rats (3.9×10^{-6}) in the current study (Figure 8).

In the earlier study (Meng et al. 1998a; Walker and Meng 2000), we also demonstrated that the maximum *Hprt* M*f*s attributable to BD exposure were 2.4-fold greater in female mice than in female rats of similar age exposed to 1250 ppm BD for 2 weeks. Similarly, in the current study, we demonstrated that the maximum *Hprt* M*f*s attributable to BD exposure were 2.0-fold greater in 4- to 5-week-old male mice than in male rats of similar age exposed to 1250 ppm BD for 2 weeks (Figure 8).

SPECIFIC AIM 3. *meso*-BDO_2 EXPERIMENTS

Stereo-Selective Synthesis of *meso*-BDO_2

One aim of the current project was to test the hypothesis that differences in the mutagenic potency of the stereochemical forms of BDO_2 might make a significant difference in the mutagenic responses to BD in mice or in species-related mutagenic effects in mice versus rats. A potentially limiting factor in planning in vivo exposures of mice and rats to *meso*-BDO_2 was finding a source of this stereoisomer in sufficient purity and quantity.

The inhalation studies in our current investigation called for nearly a deciliter of *meso*-BDO_2, and a scheme for high-yield stereoselective synthesis of this agent had to be developed (Figure 3). A range of oxygenated BD metabolites, including *meso*-BDO_2, had been synthesized by Sharpless epoxidation of 1-tosyloxy-2-hydroxy-3-butene, followed by treatment with a base (Neagu and Tapio 1993; Nieusma et al. 1998). This procedure has sufficed for the milligram quantities of *meso*-BDO_2 needed for in vitro studies (Nieusma et al. 1998; Irons et al. 2000). However, it was not practical to scale these reactions up to produce the multigram quantities needed for in vivo inhalation exposures; and a suitable alternative procedure could not be found in the literature. *meso*-BDO_2 has been produced as an isomeric mixture, generally through the epoxidation of BD (Monnier 1997) or BDO (Melnik et al. 1996) or from base-catalyzed cyclization of dichlorobutanediol (Stojanaowa-Antoszczyszyn et al. 1977).

The new high-yield procedure for *meso*-BDO_2 synthesis developed for this investigation (as described in the Methods and Study Design section) made use of established synthesis steps (Kabalka et al. 1986; Larock 1999). The greatest value of this procedure is that it allowed this highly volatile and toxic compound to be synthesized and isolated in multigram quantities with a high degree of physical and optical purity. It was also relatively economical and safe (Claffey 2002).

Effects of Exposure Levels and of Time Elapsed After Exposure on *Hprt* M*f*s in Mice and Rats Exposed to *meso*-BDO_2

The average inhalation chamber concentrations were 1.96 ± 0.54 ppm during a 3-week exposure targeted to 2 ppm *meso*-BDO_2 (6 hours/day, 5 days/week) and 3.86 ± 0.74 ppm during a 2-week exposure targeted to 4 ppm *meso*-BDO_2. Two experiments were performed simultaneously to (1) determine the change in M*f* over time in female mice and rats exposed for 3 weeks to 0 or 2 ppm *meso*-BDO_2 and (2) define the exposure–response pattern in female and male mice and rats exposed for 2 weeks to 0, 2, or 4 ppm *meso*-BDO_2.

During the 2- or 3-week active exposures and the post-exposure period, *meso*-BDO_2 exposure caused no clinical signs of toxicity or significant effects upon CEs of T cells from exposed mice and rats compared with control animals. The average CE in control and exposed female mice ($n = 69$; 52 exposed to 0 or 2 ppm for 3 weeks + 17 exposed to 0, 2, or 4 ppm for 2 weeks; Table 1) was 8.6% (range of 3.8 to 19.5%). The average CE in control and exposed female rats ($n = 75$; 60 exposed to 0 or 2 ppm for 3 weeks + 15 exposed to 0, 2, or 4 ppm for 2 weeks) was 15.4% (range of 4.6 to 39.2%). The average CE in control and exposed male mice ($n = 23$ exposed to 0, 2, or 4 ppm for 2 weeks) was 7.9% (range of 3.4 to 13.4%), which was similar to that in female mice. However, the average CE in control

and exposed male rats (n = 17) was 7.6% (range of 3.3 to 17.6%), which was lower than that in female rats. The same supplemented cell culture medium was used at this time point for the growth of splenic T cells from both male and female rats, but the microtiter dishes for each sex were placed into separate incubators. This suggests that the diminution of CEs in male but not female rats might be due to a temperature spike above 37°C in one CO_2 incubator during the 8 to 9 days allowed for phenotypic expression and growth of *Hprt* mutant colonies.

In control mice and rats, we found no significant differences between the sexes in the mean *Hprt* M*f* values; the spontaneous M*f*s in both mice ($1.5 \pm 0.7 \times 10^{-6}$) and rats ($3.9 \pm 1.1 \times 10^{-6}$) fell within the range of historical values for these strains of mice (Skopek et al. 1992, 1996; Tates et al. 1994, 1998; Walker et al. 1996, 1997, 1998; Meng et al. 1998a,b, 1999a,b) and rats (Aidoo et al. 1991, 1993; Meng et al. 1998a, 1999a,b).

Time After Exposure The effect of time elapsed after exposure on *Hprt* M*f*s was evaluated after 3-week exposures (6 hours/day, 5 days/week) of female rats and mice to 0 or 2 ppm *meso*-BDO_2. Significantly elevated *Hprt* M*f*s were observed in exposed female rats at 1 week after exposure (P = 0.004), reached a maximum value at 4 weeks after exposure ($11.7 \pm 3.5 \times 10^{-6}$ vs. $4.7 \pm 3.5 \times 10^{-6}$ in female control rats; P = 0.006), and remained high until returning to background at 9 weeks after exposure (Figure 9). After Bonferroni adjustments were made to P values, the M*f* elevations remained significant in exposed female rats at 1, 4, 5, and 6 weeks after exposure but were not significant at 2 or 7 weeks after exposure because of interanimal variation. The mutagenic effect of *meso*-BDO_2 was not as marked in female mice; the only significant elevation in M*f*s was found at 4 weeks after exposure, and it was only 2.3-fold greater than control values ($3.84 \pm 2.4 \times 10^{-6}$ vs. $1.7 \pm 3.5 \times 10^{-6}$; P = 0.046; Figure 9). These results matched well with *Hprt* M*f*s previously found at 4 weeks after exposure of female rats and mice for 4 weeks to 2 ppm (±)-BDO_2; in that study the increases in exposed rats and mice were 3.3 and 2.8 times greater, respectively, than those in control animals (Walker and Meng 2000).

Exposure Level The effects of *meso*-BDO_2 exposure level on *Hprt* M*f*s were assessed in female and male mice and rats after a 2-week exposure (6 hours/day, 5 days/week). *Hprt* M*f*s were measured at the time of peak mutagenic response for each sex of mice and rats of this age (4 to 5 weeks at start of exposure): 4 weeks after cessation of exposure for females and 5 weeks after exposure for males.

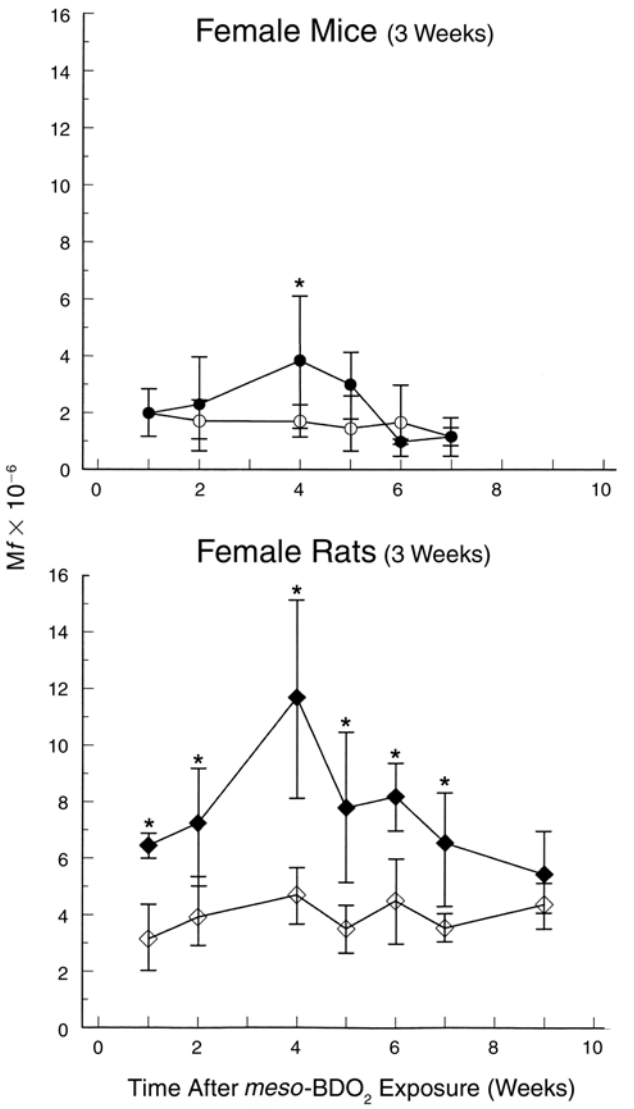

Figure 9. Time-related *meso*-BDO_2 effects on *Hprt* M*f*s in splenic T cells from female mice and rats. After 3-week exposures to 0 or 2 ppm *meso*-BDO_2, female rodents were necropsied at 1, 2, 4, 5, 6, 7, and 9 (rats only) weeks after exposure. Note the significant increase in M*f*s in mice only at 4 weeks after exposure and in rats at 1 through 7 weeks after exposure. Filled symbols indicate exposed animals. Data points are means with error bars (SD) (n = 4 or 5/group). An asterisk (★) indicates a significant difference from control values. (Specific Aim 3)

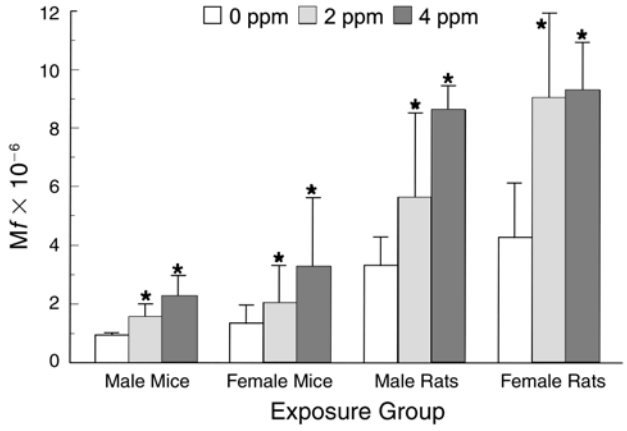

Figure 10. Exposure–responses for *Hprt* mutant splenic T cells of male and female mice and rats after exposure to *meso*-BDO_2. Groups of mice and rats were exposed to 0, 2, or 4 ppm *meso*-BDO_2 for 2 weeks. Spleens were collected 4 weeks after exposure of female animals and 5 weeks after exposure of male animals (n = 5–8 of each sex/species/exposure level). T cells were isolated and cultured under selective conditions. M*f*s for cells from male and female mice and rats exposed to 2 ppm and 4 ppm *meso*-BDO_2 were significantly increased after exposure. Data points are means with error bars (SD). An asterisk (★) indicates a significant difference from control values. (Specific Aim 3)

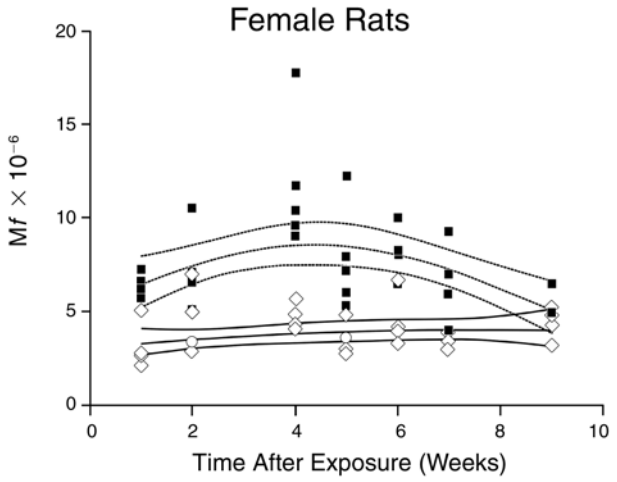

Figure 11. Curves of *Hprt* M*f* data obtained from individual female rats after a 3-week exposure to 0 or 2 ppm *meso*-BDO_2. Groups of rats were necropsied at 1, 2, 4, 5, 6, 8, and 9 weeks after exposure (n = 5/exposure group/time point). Data obtained from cells 1 to 9 weeks after exposures were used 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 this *meso*-BDO_2 exposure regimen. ◇ indicates individual control animals (0 ppm *meso*-BDO_2), lower curve and CIs; ■ indicates individual exposed animals (2 ppm *meso*-BDO_2), upper curve and CIs. (Specific Aim 3)

For female rats, exposure to 2 or 4 ppm *meso*-BDO_2 resulted in nearly identical increases in mean M*f*s at 4 weeks after exposure ($9.1 \pm 3.2 \times 10^{-6}$ for 2 ppm and $9.3 \pm 1.6 \times 10^{-6}$ for 4 ppm compared with $4.3 \pm 1.9 \times 10^{-6}$ in control animals; $P = 0.02$ and 0.003, respectively; Figure 10). For male rats exposed to 2 and 4 ppm *meso*-BDO_2, M*f*s were similar at 5 weeks after exposure ($5.6 \pm 2.9 \times 10^{-6}$ for 2 ppm and $6.2 \pm 3.3 \times 10^{-6}$ for 4 ppm compared with $3.3 \pm 1.0 \times 10^{-6}$ in control animals; $P < 0.05$). However, the exposure-induced mutagenic effects were less than those found in exposed female rats at 4 weeks after exposure (Figure 10). For female mice, exposure to 2 or 4 ppm *meso*-BDO_2 resulted in significant increases ($2.1 \pm 1.3 \times 10^{-6}$ for 2 ppm and $3.3 \pm 2.3 \times 10^{-6}$ for 4 ppm) over the control value ($1.4 \pm 0.6 \times 10^{-6}$; $P = 0.05$ and 0.026, respectively; Figure 10). For male mice exposed to 2 or 4 ppm *meso*-BDO_2, M*f*s were similar at 5 weeks after exposure ($1.6 \pm 0.4 \times 10^{-6}$ for 2 ppm and $2.3 \pm 0.6 \times 10^{-6}$ for 4 ppm compared with $1.1 \pm 0.2 \times 10^{-6}$ in control animals; $P < 0.016$). However, the exposure-induced mutagenic effects were less than those found in exposed female mice at 4 weeks after exposure (Figure 10). Within each species, the mutagenic response to exposure to 2 or 4 ppm *meso*-BDO_2 at a single point in time appeared to be greater in female animals than in males, but the differences were not significant.

Overall Mutagenicity To estimate the overall mutagenic effects of 3 weeks of exposure to 2 ppm *meso*-BDO_2 in female mice and rats, curves were fitted to individual M*f* data. The differences in the areas under the curve for *Hprt* mutant splenic T cells of exposed versus control animals of the same species represent the mutagenic potency for the given exposure in that species. Figure 11 shows representative curves and 95% Monte Carlo CIs estimated using individual *Hprt* M*f* measurements for control and exposed female rats. For this *meso*-BDO_2 exposure regimen, the estimate of mutagenic potency for female rats (29.4; 95% CI, 23.4–36.0) was approximately sevenfold greater than the estimate for female mice (4.1; 95% CI, 0.8–8.7; Table 5). Furthermore, it was notable that in female rats there was no significant difference between the mutagenic potency after 3 weeks of exposure to 2 ppm *meso*-BDO_2 and that found after 4 weeks of exposure to 2 ppm (±)-BDO_2 in a similar protocol in an earlier study (Walker and Meng 2000). Overall, these data indicate that there is no significant difference in the mutagenic potency of *meso*-BDO_2 and (±)-BDO_2 in either rats or mice (Table 5).

SPECIFIC AIM 4. BD-DIOL EXPERIMENTS

Verification of the Selectivity and Accuracy of the Method for Measuring BD-diol in Plasma or Urine

Several potentially confounding analytical questions were addressed when developing our method: (1) If BDO or BDO_2 were present in the extract, would processing the sample

Table 5. Estimated Mutagenic Potency of Exposure to BD, (±)-BDO, (±)-BDO_2, *meso*-BDO_2, and BDO-diol in Female Mice and Rats[a]

Exposure	Mutagenic Potency[b]		Comments
	Mice	Rats	
Results from Earlier Studies			
BD 62.5 ppm	10.9	NS	
BD 625 ppm	61.2	7.2	For mice, the 95% CIs for mice (49.4, 74.7) and rats (4.2, 10.5) exposed to 625 ppm BD do not overlap, which demonstrates that the mutagenic potency of BD in mice is significantly higher than in rats. For rats, the mutagenic potency estimates for exposure to 625 ppm BD and to 36 ppm BD-diol are identical (see below). This indicates that the BD-diol pathway, which leads to BDO-diol, is the major source of mutagenicity in rats exposed to BD at levels high enough to induce *Hprt* mutations.
(±)-BDO 2.5 ppm	3.9	NS	In mice, exposure to 2.5 ppm (±)-BDO produced a plasma concentration of BDO similar to that found after exposure to 62.5 ppm BD.
(±)-BDO 25 ppm	8.8	NS	In mice, exposure to 25 ppm (±)-BDO produced a plasma concentration of BDO similar to that found after exposure to 625 ppm BD.
(±)-BDO_2 2 ppm	11.2	20.9	When animals were exposed directly to (±)-BDO_2, the 95% CIs for mice (7.4, 15.7) and rats (16.3, 30.6) did not overlap; this demonstrates that the mutagenic potency is significantly higher in rats than in mice.
(±)-BDO_2 4 ppm	12.7	35.9	In mice, exposure to 3.8 ppm (±)-BDO_2 produced a plasma concentration of BDO_2 similar to that found after exposure to 62.5 ppm BD. When animals were exposed directly to (±)-BDO_2, the 95% CIs for mice (8.8, 17.1) and rats (29.0, 46.9) did not overlap; this demonstrates that mutagenic potency is significantly higher in rats than in mice.
Results from the Current Study			
meso-BDO_2 2 ppm	4.1	29.4	When animals were exposed directly to *meso*-BDO_2, the 95% CIs for mice (0.8, 8.7) and rats (23.4, 36.0) did not overlap; this demonstrates that mutagenic potency is significantly higher in rats than in mice.
BD-diol 18 ppm	7.8	Not done	In mice, exposure to 18 ppm BD-diol produced a plasma concentration of BD-diol similar to that found after exposure to 200 ppm BD. The change in M*f* over time was not tested in female rats at 18 ppm BD-diol because we anticipated that the mutagenic response would be weak.
BD-diol 36 ppm	12.5	7.2	In female mice, exposure to 36 ppm BD-diol produced a plasma concentration of BD-diol ~25% of that found after exposure to 625 ppm BD. The overlap in the 95% CIs for BD-diol–exposed mice (3.0, 14.4 at 18 ppm; and 9.9, 15.1 at 36 ppm) and rats (3.4, 11.1 at 36 ppm) shows that the mutagenic potency of metabolites derived from BD-diol are not significantly different in mice and rats. For rats, the mutagenic potency estimates for exposure to 625 ppm BD and to 36 ppm BD-diol are identical (see above). This indicates that the BD-diol pathway, which leads to BDO-diol, is the major source of mutagenicity in rats exposed to BD at levels high enough to induce *Hprt* mutations.

[a] See text for detailed description of mutagenic potency, exposure regimens, *Hprt* M*f* analysis, and data for animals exposed to *meso*-BDO_2 and BD-diol. See Walker and Meng (2000) for data for animals exposed to BD, (±)-BDO, and (±)-BDO_2. NS = not significant.

[b] Mutagenic potency is a relative value that represents the number of mutant cells passing through the spleen over the period of time 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].

lead to compound hydrolysis and result in a false high value for BD-diol? (2) Is there stereo-selectivity in the butylboranate derivatization, such that a derivatization of the racemic mixture would only result in partial derivatization?

Interference from the epoxide compounds (BDO and BDO_2) was assessed by spiking standard solutions with BD-diol alone or in combination with BDO or BDO_2. The spiked standards were processed through the entire sample extraction and analysis procedure described in the Methods section. Results for the BD-diol analysis did not change despite the addition of the epoxide compounds, which confirmed the selectivity of the assay for BD-diol.

The stereo-selectivity of the BD-diol assay was assessed by extracting and analyzing standards of the *cis* isomer, the *trans* isomer, and the racemic mixture of BD-diol (all purchased from Aldrich) and comparing the responses. There was no statistical difference between the two stereoisomers and the racemic mixture in the amounts of measured BD-diol. Thus, *cis* and *trans* isomers were measured as total BD-diol in plasma and urine samples.

Effects of BD or BD-diol Exposure Level on Plasma Concentrations of BD-diol in Mice and Rats

The goal of the limited metabolism studies performed here with mice and rats exposed to BD or BD-diol (Inhalation Studies A, B, D, and E) was to determine the levels of exposure to BD-diol that would result in BD-diol plasma concentrations equivalent to those that result from exposures to 62.5 and 625 ppm BD. Results in female mice are of particular interest because they are most sensitive to cancer induction; information about formation and excretion of metabolites in this sex and species may help assess the relationship between BD metabolism and cancer susceptibility. In the inhalation studies conducted for Specific Aim 4, the 6-hour exposures to BD (Inhalation Studies A and D) and the 6-hour (Inhalation Studies B and E) and 4-week (Inhalation Studies C and F) exposures to BD-diol described below caused no clinical signs of toxicity.

Effects of BD Exposure Level on Plasma Concentrations of BD-diol For Inhalation Studies A and D, the average concentrations for the 6-hour BD exposures were 67.1 ± 4.0 ppm, 203 ± 33 ppm, 638 ± 23 ppm, and 1242 ± 51 ppm for the respective target exposures of 62.5, 200, 625, and 1250 ppm BD. In plasma separated from blood collected from animals immediately (3 minutes) after 6-hour exposures to (targeted) 0, 62.5, 625, or 1250 ppm BD, BD-diol was not found in the plasma of control mice and rats nor in that of mice or rats exposed to 62.5 ppm BD (Figure 12). However, BD-diol accumulated in the plasma of all groups of exposed rodents in a sublinear manner (i.e., with positive curvature) at the two higher levels of exposure.

At 625 ppm BD, plasma concentrations of BD-diol in female and male mice were significantly higher than those in female and male rats ($P = 0.01$; Figure 12). Also at 625 ppm BD, plasma concentrations in female mice were higher than those in male mice ($P < 0.001$).

At 1250 ppm BD, plasma concentrations of BD-diol were significantly higher in male rodents than in female rodents ($P < 0.01$); the concentrations of BD-diol in male mice and male rats were not significantly different (Figure 12). The pattern of BD-diol accumulation in the plasma of female mice exposed to 625 or 1250 ppm BD was distinctly different from that of the other groups of concurrently exposed rodents. Therefore the plasma samples from these female mice were reanalyzed and similar values were obtained again. This suggests that a plateau in BD-diol plasma concentrations may occur in this dose range in female mice.

In a follow-up 6-hour exposure of only female mice and rats to an intermediate level of 200 ppm BD (Inhalation Study D), BD-diol was detected in the plasma of both groups at relatively low concentrations (compared with those detected at 625 and 1250 ppm BD). But the BD-diol concentrations in female mice (0.173 ± 0.068 µg/mL) were significantly higher than those in female rats (0.073 ± 0.025 µg/mL; $P = 0.006$).

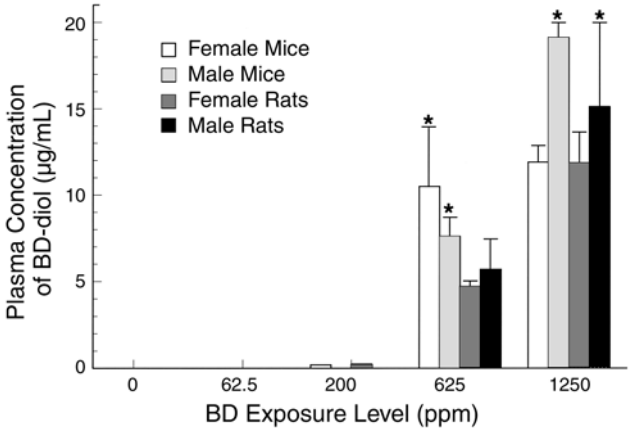

Figure 12. Effect of BD exposure level on plasma concentrations of BD-diol in female and male mice and rats. Groups of mice and rats were necropsied immediately after a single 6-hour nose-only exposure to 0, 62.5, 200 (female animals only), 625, or 1250 ppm BD ($n = 5$ or 6 of each sex/species/exposure level). Plasma concentrations of BD-diol were undetectable in control animals and animals exposed to 62.5 ppm BD. Data points are means with error bars (SD). At 625 ppm BD, plasma concentrations of BD-diol in female mice were significantly higher than those in male mice (see text). An asterisk (✶) for mice (both sexes) exposed to 625 ppm BD indicates significantly higher plasma concentrations of BD-diol than exposed rats; an asterisk (✶) for male animals (both species) exposed to 1250 ppm BD indicates significantly higher plasma concentrations of BD-diol compared with exposed female animals. (Specific Aim 4, Inhalation Studies A and D)

Blood was also collected from these groups of female rodents at 2, 4, 6, and 24 hours after exposure to 200 ppm BD (Inhalation Study D) in order to define the half-life of BD-diol in plasma. Unfortunately, these time points were not wisely chosen because it turned out that BD-diol was cleared from the plasma of both mice and rats in less than 2 hours. In retrospect, the quick plasma clearance of BD-diol in BD-exposed female rodents was consistent with the results of an earlier experiment by Kemper and associates (1998). In that study, BD-diol was injected intraperitoneally in male B6C3F1 mice and was eliminated from plasma in less than 125 minutes after a dose of 25 mg/kg.

Effects of BD-diol Exposure Level on Plasma Concentrations of BD-diol Effects were explored in two independent 6-hour Inhalation Studies (B and E) and in two 4-week Inhalation Studies (C and F).

In Inhalation Study B, female and male mice and rats were exposed for 6 hours to 0, 6, or 18 ppm BD-diol, and in Inhalation Study E, female mice were exposed to 0, 24, or 36 ppm BD-diol.

After exposures to 6 or 18 ppm BD-diol, plasma concentrations of BD-diol increased in an exposure-related manner; there were no remarkable species or sex differences (Figure 13). Plasma concentrations were roughly linear after exposure to 6 or 18 ppm BD-diol (i.e., about threefold higher concentrations of metabolite/mL plasma at 18 ppm than at 6 ppm). In female mice exposed to 18 or 24 ppm, however, BD-diol accumulated in a sublinear manner with increasing exposure levels (i.e., the plasma concentration of BD-diol increased twofold as the exposure level was raised from 18 to 24 ppm and then increased 4.4-fold as the exposure level was raised from 24 to 36 ppm).

In Inhalation Study C, female mice and rats were exposed for 4 weeks (6 hours/day, 5 days/week) to 0, 6, or 18 ppm BD-diol; and in Inhalation Study F, female mice and rats and male mice were exposed for 4 weeks (6 hours/day, 5 days/week) to 0 or 36 ppm BD-diol. (The average inhalation chamber concentrations for the 4-week BD-diol exposure periods [Inhalation Study C and F] were 6.0 ± 0.7 ppm, 16.5 ± 0.7 ppm, and 33.4 ± 4.7 ppm for the respective target exposures of 6, 18, and 36 ppm BD-diol.)

The BD-diol plasma concentrations after 6-hour exposures to 6 or 18 ppm BD-diol (Inhalation Study B) were compared with those found after 4-week exposures (6 hours/day, 5 days/week) to 6 or 18 ppm BD-diol (Inhalation Study C; Figure 14). At first glance, plasma concentrations of BD-diol appeared to be somewhat higher in female rats after a single 6-hour exposure to 6 or 18 ppm BD-diol than after repeated exposures (4 weeks). However, there was more interanimal variation in the single-exposure groups of rats, and there was no statistical difference between the average values obtained after single or multiple exposures. The plasma concentrations of BD-diol were quite similar after single and multiple exposures, demonstrating that repeated BD-diol exposures do not affect the clearance of this metabolite.

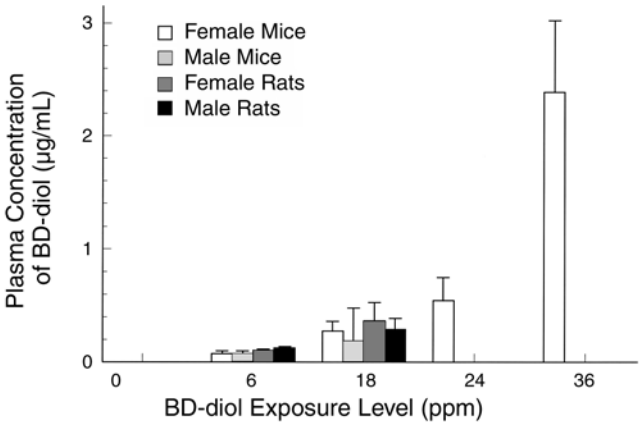

Figure 13. Effects of BD-diol exposure on plasma concentrations of BD-diol in female and male mice and rats. All four groups of mice and rats underwent a single 6-hour nose-only exposure to 0, 6, or 18 ppm BD-diol; only female mice were exposed in the same manner to 24 or 36 ppm BD-diol (n = 6 of each sex/species/exposure level). Data points are means with error bars (SD). (Specific Aim 4, Inhalation Studies B and E)

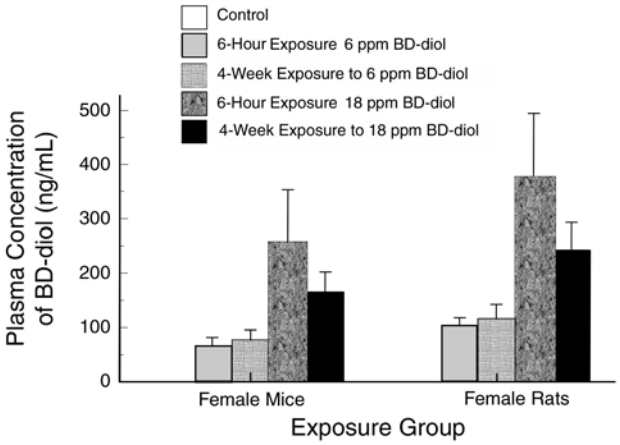

Figure 14. Comparison of BD-diol plasma concentrations in female mice and rats after single or repeated exposures to BD-diol. Groups of mice and rats were necropsied either immediately after a single 6-hour exposure or immediately after repeated exposures for 4 weeks (6 hours/day, 5 days/week) to 0, 6, or 18 ppm BD-diol (n = 6/species/exposure level). Data points are means with error bars (SD). (Specific Aim 4, Inhalation Studies B and C)

Comparison of Plasma BD-diol Concentrations from Exposures to BD and BD-diol In order to compare the relative mutagenic potency of the BD-diol pathway in BD mutagenicity, it was important to identify exposure levels of BD-diol and BD that would result in the same plasma concentrations of BD-diol in female mice. (We used female mice because previous comparisons of the mutagenic potencies of inhalation exposures to BD, BDO, and BDO_2 were predicated on achieving equivalent circulating blood levels of these epoxide metabolites in female mice, the sex and species of rodents most sensitive to BD-induced carcinogenesis [Walker and Meng 2000].) This undertaking was hampered by the fact that BD-diol proved difficult to vaporize for inhalation exposures and that 36 ppm BD-diol proved to be the highest exposure level we could achieve for evaluating *Hprt* M*f*s after repeated (4-week) exposures of mice and rats. Figure 15 shows that the plasma concentrations of BD-diol found after single (6-hour) or repeated (4-week) exposures of female mice to 18 ppm BD-diol were basically equivalent to those produced by exposure of female mice to 200 ppm BD (similar results were obtained in female rats; data not shown). However, when the exposure level of BD-diol was doubled to 36 ppm, the plasma concentrations of BD-diol were only 25% of the plasma levels achieved in female mice exposed to 625 ppm BD. Thus, the problem in vaporizing BD-diol prevented us from achieving our stated goal of determining the mutagenicity of BD-diol alone when present in plasma at concentrations equivalent to those achieved in female mice exposed to 625 ppm BD.

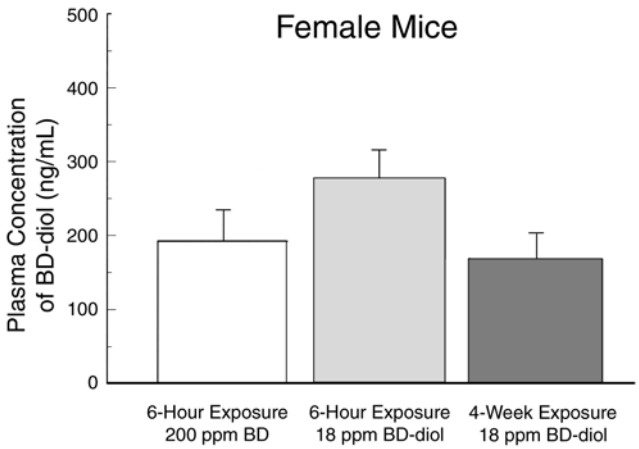

Figure 15. Comparison of BD-diol plasma concentrations in female mice after single or repeated exposures to BD or BD-diol. Groups of mice were necropsied immediately after a single 6-hour exposure to 0 or 200 ppm BD, immediately after a single 6-hour exposure to 0 or 18 ppm BD-diol, or immediately after repeated exposures for 4 weeks (6 hours/day, 5 days/week) to 0 or 18 ppm BD-diol (n = 6 animals/group). Data points are means with error bars (SD). Detectable concentrations of BD-diol were not found in control mice. (Specific Aim 4, Inhalation Studies B, C, and D)

Urinary Excretion of BD Metabolites

Groups of female mice and rats exposed to 200 ppm BD for 6 hours (Inhalation Study D) were placed in metabolic cages for the 24-hour period after exposure primarily to determine if measurable amounts of BD-diol were excreted in the urine of either species. Samples from female mice were not analyzed because the urine was contaminated with mouse droppings due to filters in the metabolic cages that were improperly fitted. In exposed female rats, only minor concentrations of BD-diol (0.041 ± 0.002 µg/mL) were found in urine compared with the concentrations of M1 (21.4 ± 0.9 µg/mL) and M2 (44.3 ± 19.9 µg/mL). However, the minor amounts of BD-diol found in the urine of these female F344 rats was consistent with an earlier demonstration by Nauhaus and coworkers (1996) that, in BD-exposed male Sprague-Dawley rats and B6C3F1 mice, BD-diol comprised 5% and 3%, respectively, of the total metabolites excreted.

Effects of Time Elapsed After BD-diol Exposure on *Hprt* M*f*s in Mice and Rats

***CEs and Spontaneous Background* Hprt *Mf*s** The 4-week BD-diol exposures had no significant effects on CEs of T cells from exposed mice or rats compared with control animals. Within each species, there were no significant sex-related differences in CEs between control and BD-diol–exposed mice or rats after 4 weeks of exposure. The mean CEs in control mice (n = 50 females and 35 males) and BD-diol–exposed mice (n = 50 females and 51 males) were 9.5% (range 4.2 to 14.7%) and 8.2% (range 5.2 to 15.2%), respectively. The mean CEs in control rats (n = 50 females and 10 males) and BD-diol–exposed rats (n = 50 females and 20 males) were 18.1% (range 9.1 to 28.9%) and 17.9% (range 7.9 to 34.0%), respectively.

Also, there were no significant sex-related differences in the mean *Hprt* M*f* values between control mice and rats; the spontaneous M*f*s in mice ($1.6 \pm 0.5 \times 10^{-6}$) and rats ($3.1 \pm 1.1 \times 10^{-6}$) both agreed with those found in control animals in the *meso*-BDO_2 inhalation experiment (Specific Aim 3) and with historical control values for these strains of mice (Skopek et al. 1992, 1996; Tates et al. 1994, 1998; Walker et al. 1996, 1997, 1998; Meng et al. 1998a,b, 1999a,b) and rats (Aidoo et al. 1991, 1993; Meng et al. 1998a, 1999a,b).

***Time After Exposure and* Hprt *Mf*s** The effects of time elapsed after exposure were evaluated in female mice and rats after 4-week exposures to 0, 6, or 18 ppm BD-diol (Inhalation Study C). Significantly elevated *Hprt* M*f*s were found in female mice at 5.5 weeks after exposure to 6 or

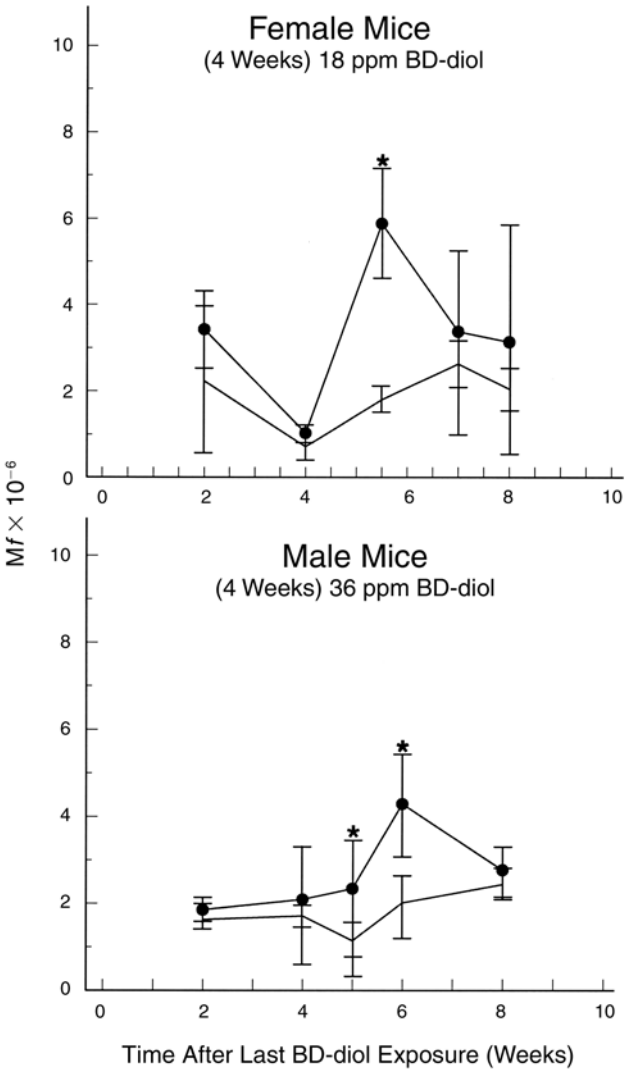

Figure 16. Time-related effects of BD-diol on *Hprt* M*f*s in splenic T cells after a 4-week exposure to 18 ppm BD-diol (female mice) or to 36 ppm BD-diol (male mice). Groups of female mice were necropsied at 2, 4, 5.5, 7, and 8 weeks after last exposure; groups of male mice were necropsied at 2, 4, 5, 6, and 8 weeks after last exposure. Note the significant increase in M*f*s in female mice only at 5.5 weeks after exposure, and in male mice at 5 and 6 weeks after exposure. Filled symbols indicate exposed animals. Data points are means with error bars (SD) (n = 5 females and 5–8 males/exposure group/time point). An asterisk (★) indicates a significant difference from control values. (Specific Aim 4, Inhalation Studies C and F)

18 ppm BD-diol (mean M*f*s = 3.1 ± 1.1 × 10^{-6} and 5.9 ± 1.3 × 10^{-6}, respectively) compared with controls (1.8 ± 0.4 × 10^{-6}; P = 0.032 and 0.001, respectively; Figure 16, upper graph). There was an apparent elevation in *Hprt* M*f*s at 5.5 weeks after exposure in female rats exposed to 18 ppm BD-diol, but the increase was not significant because of the presence of two higher-than-typical M*f* values in the 5 control female rats (data not shown).

Male mice and rats were exposed to BD-diol mainly for the purpose of collecting T-cell mutants for future molecular analyses. In male mice necropsied at 4 weeks after exposure to 6 or 18 ppm BD-diol (Inhalation Study C), *Hprt* M*f*s were significantly higher than background (mean M*f* = 0.44 ± 0.20 × 10^{-6}) after exposure (mean M*f*s = 1.28 ± 0.73 × 10^{-6} and 1.27 ± 0.73 × 10^{-6}, respectively; P values = 0.009 and 0.038, respectively).

For male mice and rats, *Hprt* M*f*s were significantly elevated at 4 weeks after exposure to 6 or 18 ppm BD-diol (mean M*f*s = 1.28 ± 0.73 × 10^{-6} and 1.27 ± 0.73 × 10^{-6}, respectively; P = 0.009 and 0.038, respectively) compared with control values (mean M*f* = 0.44 ± 0.20 × 10^{-6}; data not shown). Significantly elevated *Hprt* M*f*s were also found at 4 weeks after exposure of male rats, but only to 18 ppm BD-diol (mean M*f* = 2.26 ± 0.66 × 10^{-6}; P = 0.033) compared with controls (mean M*f* = 1.37 ± 0.34 × 10^{-6}).

A follow-up experiment was performed using a higher exposure level of BD-diol (36 ppm for 4 weeks; Inhalation Study F), in part to confirm the positive finding of mutagenicity in male and female mice exposed to 6 or 18 ppm, as described above, and to determine if BD-diol exposure was indeed mutagenic in female rats.

For male mice, significantly increased *Hprt* M*f*s were found at 5 and 6 weeks after exposure to 36 ppm BD-diol (mean M*f*s in exposed mice were 2.34 ± 0.91 × 10^{-6} and 4.29 ± 1.06 × 10^{-6} at 5 weeks and 6 weeks respectively, compared with control values of 1.14 ± 0.84 × 10^{-6} and 2.01 ± 0.75 × 10^{-6}; Figure 16, lower graph). After Bonferroni adjustments to P values were made, however, the elevation in M*f* remained significant only at the time of peak mutagenic response, which was 6 weeks after exposure.

For female mice, significantly increased *Hprt* M*f*s were also observed (P = 0.023), reached a maximum value at 6 weeks after exposure (mean M*f* = 6.12 ± 4.22 × 10^{-6} vs. 1.65 ± 1.17 × 10^{-6} in controls; P = 0.008), and returned to background values at 8 weeks after exposure (Figure 17, upper graph). After Bonferroni adjustments to P values were made, the elevation in M*f* remained significant at 5 and 6 weeks after exposure but not at 4 weeks.

For female rats exposed to BD-diol, increases in *Hprt* M*f*s reached a peak at 5 weeks after exposure (mean M*f* = 5.62 ± 0.40 × 10^{-6} versus 2.27 ± 0.52 × 10^{-6} in controls), remained elevated at 6 weeks after exposure, and returned to background levels at 8 weeks after exposure (Figure 17, lower graph). After Bonferroni adjustments to P values were made, the elevation in M*f* in exposed female rats remained significant only at the time of peak mutagenic response, which was 5 weeks after exposure.

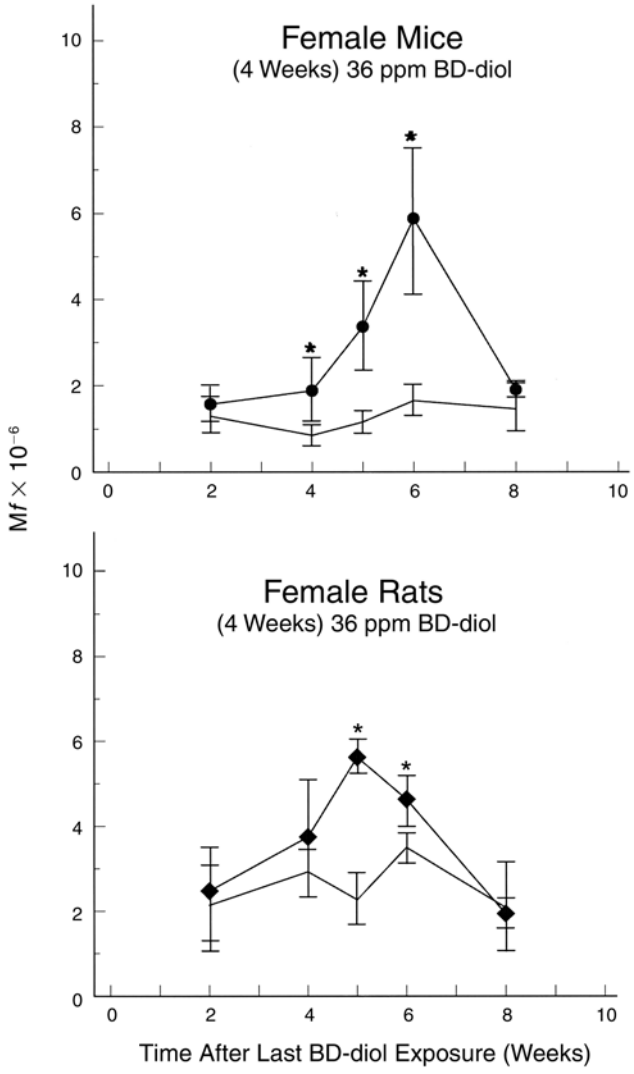

Figure 17. Time-related effect of BD-diol on *Hprt* M*f*s in splenic T cells from female mice and rats after a 4-week exposure to 0 or 36 ppm BD-diol. Groups of animals were necropsied at 2, 4, 5, 6, and 8 weeks after exposure. Note the significant increase in M*f*s in mice at 4, 5, and 6 weeks after exposure and in rats at 5 and 6 weeks after exposure. Filled symbols indicate exposed animals. Data points are means with error bars (SD) (n = 5/species/exposure level/time point). An asterisk (★) indicates a significant difference from control values. (Specific Aim 4, Inhalation Study F)

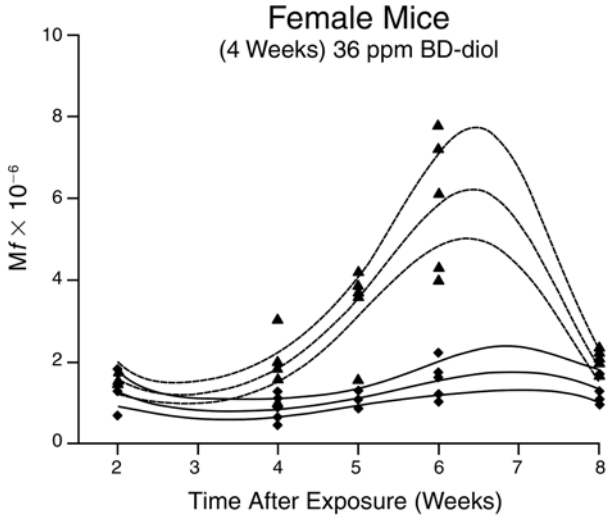

Figure 18. Curves of *Hprt* M*f* data obtained from individual female mice after a 4-week exposure to 0 or 36 ppm BD-diol. Groups of mice were necropsied at 2, 4, 5, 6, and 8 weeks after exposure (n = 5/exposure level/time point). Data obtained from cells 2 to 8 weeks after exposure were used to derive 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 of this BD-diol exposure regimen (see Table 1). ▲ indicates an individual exposed animal (36 ppm BD-diol) on the upper curve and CIs. ◆ indicates an individual control animal (0 ppm BD-diol) on the lower curve and CIs. (Specific Aim 4, Inhalation Study F)

Mutagenic Potency of BD-diol Exposures in Splenic T Cells from Mice and Rats

To estimate the overall mutagenic response to 4-week exposures, curves were fitted to individual M*f* data for female rats and male mice exposed to 36 ppm BD-diol (Inhalation Study F) and for female mice exposed to 6, 18, or 36 ppm BD-diol (Inhalation Studies C and F). The areas under the curves for exposed and control animals represent the mutagenic potency of a given exposure in the given species and sex. For the rodents exposed to 36 ppm BD-diol, the estimates of mutagenic potency for male mice (6.30; 95% Monte Carlo CI, 2.94–9.92) and for female rats (7.21; 95% CI, 3.43–11.10) were not significantly different (Table 5). For the exposed female mice, the mutagenic potency estimates (and 95% CIs) were 3.86 (0.31–7.35), 7.84 (2.93–14.35), and 12.53 (9.99–15.13) for exposures to 6, 18, and 36 ppm BD-diol, respectively (Table 5). (Figure 18 shows representative curves and 95% CIs estimated using individual *Hprt* M*f* measurements for female mice exposed to 36 ppm BD-diol and control mice.) Comparisons of the mutagenic potency estimates in Table 5 showed that the overall effects in male mice and female rats exposed to 36 ppm BD-diol were essentially the same as those found in female mice exposed to 18 ppm BD-diol. The mutagenic potency (12.53) of a 36-ppm BD-diol exposure in female mice was higher than the mutagenic potency (7.21) of an 18-ppm BD-diol exposure in female rats.

The pattern of mutagenic effects as related to exposure level was investigated by comparing the mutagenic potency estimates for female mice exposed to 6, 18, or 36 ppm BD-diol (Inhalation Studies C and F). The resulting exposure–response plot of mutagenic potency against exposure level suggested that mutagenic effects would reach a plateau at exposure levels exceeding 36 ppm BD-diol

(Figure 19, top panel). This observation was supported by comparing the efficiency of induction of mutagenic effects [(mutagenic potency) ÷ (ppm BD-diol) = relative efficiency in producing *Hprt* mutations] at the three BD-diol exposure levels (Figure 19, bottom panel). Normalization against the calculated value at 36 ppm BD-diol gives a mutagenic efficiency ratio of 1.85, 1.25, and 1.0 for the relative degree of effects per unit exposure at 6, 18, and 36 ppm BD-diol, respectively.

SPECIFIC AIM 5. BD MUTATION-SPECTRA EXPERIMENTS

Hprt M*f*s in Splenic T Cells of Control and BD-Exposed Male Mice and Rats

Hprt mutants were collected from male mice and rats that were used in Specific Aim 2 to investigate sex-related differences in mutagenic responses either 5 weeks (mice) or 4 weeks (rats) after a 2-week inhalation exposure (6 hours/day, 5 days/week) to 0 or 1250 ppm BD. These were the time points at which maximum BD-induced *Hprt* M*f*s would be observed in splenic T cells for rodents of this age (4–5 weeks of age at the beginning of exposure), as demonstrated in previous work in our laboratory (Meng et al. 1998b). *Hprt* mutants were also collected from control male mice and rats used for Specific Aims 3 and 4 (and necropsied at the same times after exposure) to increase the number of clones available for molecular analyses of spontaneous mutations.

The mean *Hprt* M*f* in BD-exposed male mice ($9.2 \pm 3.3 \times 10^{-6}$; $n = 30$) was 6.2-fold greater than the mean control value ($1.5 \pm 0.8 \times 10^{-6}$; $n = 20$; $P < 0.001$), such that approximately 84% of the *Hprt* mutant clones expanded for molecular analyses would be expected to have BD-induced mutations. The mean *Hprt* M*f* in BD-exposed male rats ($8.1 \pm 2.8 \times 10^{-6}$; $n = 15$) was 2.6-fold greater than the mean control value ($3.1 \pm 1.0 \times 10^{-6}$; $n = 12$; $P < 0.001$), such that approximately 62% of the *Hprt* mutant clones expanded for molecular analyses would be expected to have BD-induced mutations.

RT–PCR and cDNA Sequence Analysis of Mutants from Control and BD-Exposed Mice

A total of 145 *Hprt* mutant T-cell isolates from control male mice were processed for colony expansion, and 111 clones (77%) were expanded to more than 5×10^5 cells. The percentage of mutant isolates successfully expanded was very similar to that observed in our previous work (76%; Meng et al. 1998a), which established conditions for expanding mouse *Hprt* mutant colonies. The 111 expanded mutant clones from control mice were analyzed by RT–PCR; 54 mutants yielded cDNA products visible on 8% polyacrylamide gels. DNA sequencing showed that, of the 54 cDNA products, 5 had wild-type *Hprt* cDNA sequences and 49 had base substitutions, frameshifts, exon exclusions, or deletions (Table 6). Of the 49 mutations,

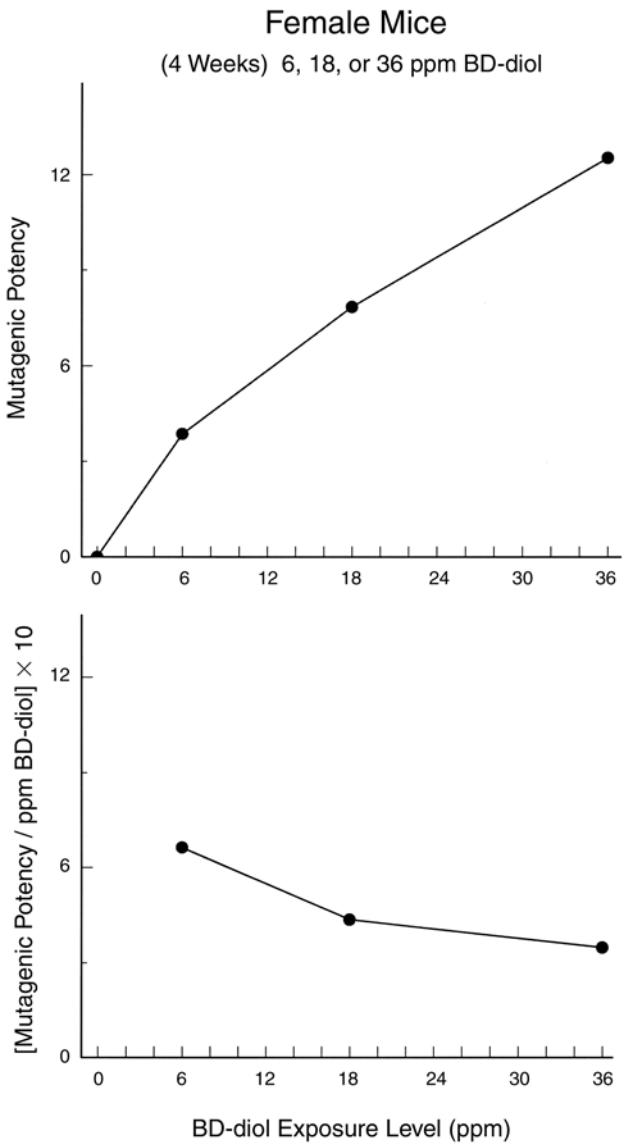

Figure 19. Exposure-related mutagenic effects of BD-diol exposure expressed as mutagenic potency and as efficiency of *Hprt* mutation induction. Female mice were exposed to 0, 6, 18, or 36 ppm BD-diol for 4 weeks and necropsied at 2, 4, 5, 6, or 8 weeks after exposure. Data obtained from cells 2 to 8 weeks after exposure were used to derive 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 of each BD-diol exposure level. Top, mutagenic potency values plotted against BD-diol exposure levels; bottom, mutagenic potency values divided by the BD-diol exposure levels plotted against BD-diol exposure levels. (Specific Aim 4, Inhalation Studies C and F)

Table 6. RT–PCR and cDNA Sequence Analysis of *Hprt* Mutant Clones Isolated from Male Mice After a 2-Week Exposure to 0 or 1250 ppm BD (Specific Aim 5)[a]

Mutation Type and Animal Number	Position[b]	Mutation[c]	Sequence Context[d]	Amino Acid Change
Base Substitutions				
Control mice				
MCM3-2	28	A → G	GTG **A**TT AGC	Ile → Val
MCM3-7	28	A → G	GTG **A**TT AGC	Ile → Val
MCM7-3	70	A → G	TGT **A**TA CCT	Ile → Val
MCM12-8	131	A → C	ATG G**A**C AGG	Asp → Ala
MCM8-8	150	T → C	CTT GC**T** CGA	Ala → Ala
MCM8-10	230	A → C	GCT G**A**C CTG	Asp → Ala
MCM6-9	259	A → C	AAT **A**GT AAT	Arg → Arg
MCM10-3	259	A → C	AAT **A**GT AAT	Arg → Arg
MCM10-11	271	A → G	GAT **A**GA TCC	Arg → Gly
MCM11-1	271	A → G	GAT **A**GA TCC	Arg → Gly
MCM6-5	274	T → C	AGA **T**CC ATT	Ser → Pro
MCM4-2	298	A → G	TTT **A**TC AGA	Ile → Val
MCM9-1	313	T → C	AGC **T**AC TGT	Tyr → His
MCM6-4	362	A → T	GAT G**A**T CTC	Asp → Val
MCM13-1	398	T → C	ATT G**T**T GAA	Val → Ala
MCM9-15	536	T → C	TTT G**T**T GGA	Val → Ala
MCM13-4	559	T → G	AAG **T**TT GTT	Phe → Val
MCM1-15	566	T → G	GTT G**T**T GGA	Val → Gly
MCM6-8	571	T → A	GGA **T**AT GCC	Tyr → Asn
MCM5-2	592	T → C	GAG **T**AC TTC	Tyr → His
MCM16-1	604	T → C	GAT **T**TG AAT	Leu → Leu
MCM13-5	605	T → C	GAT T**T**G AAT	Leu → Ser
MCM8-4	631	A → G	GAA **A**CT	Thr → Ala
MCM5-2	79	C → T	AAT **C**AT TAT	His → Tyr
MCM6-1	79	C → T	AAT **C**AT TAT	His → Tyr
MCM14-2	86	C → T	TAT G**C**C GAG	Ala → Val
MCM1-7	213	C → G	GGG GG**C** TAT	Gly → Gly
MCM1-16	213	C → G	GGG GG**C** TAT	Gly → Gly
MCM7-2	287	C → T	ATG A**C**T GTA	Thr → Ile
MCM14-1	292	G → A	GTA **G**AT TTT	Asp → Asn
MCM10-10	325	C → T	GAT **C**AG TCA	Gln → Stop
MCM12-1	325	C → T	GAT **C**AG TCA	Gln → Stop
MCM7-9	416	C → A	GAC A**C**T GGT	Thr → Asn
MCM1-10	493	G → T	CTG **G**TG AAA	Val → Leu
MCM1-1	568	G → A	GTT **G**GA TAT	Gly → Glu

Table continues next page.

[a] Exposed under Specific Aim 2 protocol; necropsy at 5 weeks after exposure.

[b] Numbering of the mouse *Hprt* gene according to Melton et al. 1984.

[c] The mutation in the nontranscribed strand is reported.

[d] The mutated base is in boldface and underlined. A series of boldfaced and underlined bases indicates a site of possible base deletion or insertion.

Table 6 (*Continued*). RT–PCR and cDNA Sequence Analysis of *Hprt* Mutant Clones Isolated from Male Mice After a 2-Week Exposure to 0 or 1250 ppm BD (Specific Aim 5)[a]

Mutation Type and Animal Number	Position[b]	Mutation[c]	Sequence Context[d]	Amino Acid Change
Base Substitutions (*continued*)				
BD-exposed mice				
MTM7-1	1	A → T	**A**TG CCG	Met → Leu
MTM8-2	1	A → T	**A**TG CCG	Met → Leu
MTM15-4	1	A → T	**A**TG CCG	Met → Leu
MTM21-1	1	A → T	**A**TG CCG	Met → Leu
MTM21-12	1	A → T	**A**TG CCG	Met → Leu
MTM21-17	1	A → T	**A**TG CCG	Met → Leu
MTM21-21	2	T → A	**AT**G CCG	Met → Lys
MTM12-4	41	A → C	GAT G**A**A CCA	Glu → Ala
MTM8-6	133	A → G	GAC **A**GG ACT	Arg → Ala
MTM18-5	194	T → C	GCC C**T**C TGT	Leu → Pro
MTM7-6	217	A → T	TAT **A**AG TTC	Lys → Stop
MTM14-4	221	T → G	AAG T**T**C TTT	Phe → Cys
MTM19-2	313	T → C	AGC **T**AC TGT	Tyr → His
MTM20-7	314	A → T	AGC T**A**C TGT	Tyr → Phe
MTM21-7	387	T → G	AAG AA**T** GTC	Asn → Lys
MTM8-1	428	T → G	ACA A**T**G CAA	Met → Arg
MTM3-4	449	T → G	CTG G**T**T AAG	Val → Gly
MTM16-3	491	T → G	TTG C**T**G GTG	Leu → Arg
MTM19-1	604	T → G	GAT **T**TG AAT	Leu → Val
MTM7-2	22	G → T	AGC **G**TC GTG	Val → Phe
MTM20-9	32	G → C	ATT A**G**C GAT	Ser → Thr
MTM20-5	40	G → C	GAT **G**AA CCA	Glu → Gln
MTM12-4	47	G → T	CCA G**G**T TAT	Gly → Val
MTM21-3	58	G → C	CTA **G**AT TTG	Asp → His
MTM20-3	88	G → A	GCC **G**AG GAT	Glu → Lys
MTM21-20	88	G → A	GCC **G**AG GAT	Glu → Lys
MTM23-2	112	C → G	ATT **C**CT CAT	Pro → Ala
MTM20-1	162	G → A	GTC AT**G** AAG	Met → Ile
MTM7-9	134	G → C	GAC A**G**G ACT	Arg → Thr
MTM8-8	211	G → A	GGG **G**GC TAT	Gly → Ser
MTM21-10	211	G → C	GGG **G**GC TAT	Gly → Ser
MTM22-10	211	G → C	GGG **G**GC TAT	Gly → Ser

Table continues next page.

[a] Exposed under Specific Aim 2 protocol; necropsy at 5 weeks after exposure.

[b] Numbering of the mouse *Hprt* gene according to Melton et al. 1984.

[c] The mutation in the nontranscribed strand is reported.

[d] The mutated base is in boldface and underlined. A series of boldfaced and underlined bases indicates a site of possible base deletion or insertion.

Table 6 (*Continued*). RT–PCR and cDNA Sequence Analysis of *Hprt* Mutant Clones Isolated from Male Mice After a 2-Week Exposure to 0 or 1250 ppm BD (Specific Aim 5)[a]

Mutation Type and Animal Number	Position[b]	Mutation[c]	Sequence Context[d]	Amino Acid Change
Base Substitutions (*continued*)				
BD-exposed mice (*Continued*)				
MTM23-4	227	C → T	TTT G**C**T GAC	Ala → Val
MTM7-16	229	G → T	GCT **G**AC CTG	Asp → Tyr
MTM23-6	232	C → T	GAC **C**TG CTG	Leu → Leu
MTM3-7	302	G → A	ATC A**G**A CTG	Arg → Lys
MTM12-20	430	C → A	ATG **C**AA ACT	Gln → Lys
MTM3-8	454	C → T	AAG **C**AG TAC	Gln → Stop
MTM6-6	529	G → A	CCA **G**AC TTT	Asp → Asn
MTM13-9	606	G → T	GAT TT**G** AAT	Leu → Phe
Insertions				
Control mice				
MCM1-9	36–37	+ G	GAT **G** GAT	Frameshift
MCM1-11	36–37	+ G	GAT **G** GAT	Frameshift
MCM1-1	41–42	+ G	GA**G**A CCA	Frameshift
BD-exposed mice				
MTM21-6	27–28	+ C	GTG **C** ATT	Frameshift
MTM13-4	98–102	+ A	G**AA AAAA** GTG	Frameshift
MTM22-11	207–212	+ G	AA**G GGGG G**CC	Frameshift
MTM20-11	515–516	+ C	AGT GT**C**T GGA	Frameshift
MTM21-16	529–531	+ A	CCA G**AA**C TTT	Frameshift
MTM14-2	530–532	+ C	CCA GA**CC** TTT	Frameshift
MTM19-7	553–554	+ T	CCA G**T**AC AAG	Frameshift
Deletions				
Control mice				
MCM1-3	8–10	− C	CCG A**CC C**GC	Frameshift
MCM1-4	8–10	− C	CCG A**CC C**GC	Frameshift
MCM1-1	43–549	− 507 bp	GAA **CC … T** CCA	In-frame deletion
MCM3-6	466–469	− A	CCC **AAA A**TG	Frameshift
MCM3-8	466–469	− A	CCC **AAA A**TG	Frameshift
MCM3-9	509	− G	TCT C**G**A AGT	Frameshift
BD-exposed mice				
MTM22-1	16–18	− C	AGT **CCC** AGC	Frameshift
MTM19-2	58	− G	CTA **G**AT TTG	Frameshift
MTM21-4	58	− G	CTA **G**AT TTG	Frameshift
MTM23-12	207–212	− G	AA**G GGG GG**C	Frameshift
MTM22-4	294–297	− T	GA**T TTT** ATC	Frameshift
MTM21-29	462–465	− C	AG**C CCC** AAA	Frameshift
MTM23-1	496–499	− A	GTG **AAA A**GG	Frameshift
MTM21-8	553	− G	CCA **G**AC AAG	Frameshift
MTM13-1	629–631	− A	AGT G**AA A**CT	Frameshift

Table continues next page.

[a] Exposed under Specific Aim 2 protocol; necropsy at 5 weeks after exposure.

[b] Numbering of the mouse *Hprt* gene according to Melton et al. 1984.

[c] The mutation in the nontranscribed strand is reported.

[d] The mutated base is in boldface and underlined. A series of boldfaced and underlined bases indicates a site of possible base deletion or insertion.

Table 6 (*Continued*). RT–PCR and cDNA Sequence Analysis of *Hprt* Mutant Clones Isolated from Male Mice After a 2-Week Exposure to 0 or 1250 ppm BD (Specific Aim 5)[a]

Mutation Type and Animal Number	Position[b]	Mutation[c]	Sequence Context[d]	Amino Acid Change
Complex Mutations				
Control mice				
No complex mutations observed				
BD-exposed mice				
MTM21-18	bp 487–571 replaced by 50 bases in a noncoding region, exon 1, and exon 2			
MTM17-2	606	G → TT	GAT TT**G** AAT	Frameshift
MTM20-13	630-631	AA → TT	AGT GA**A A**CT	GluThr → AspSer
Exon Exclusions				
Control mice				
MCM1-8	Exons 2 and 3 missing		Splicing error	
MCM1-23	Exons 2 and 3 missing		Splicing error	
MCM2-1	Exon 1 missing		Splicing error	
MCM2-2	Exon 1 missing		Splicing error	
MCM10-2	Exon 3 missing		Splicing error	
BD-exposed mice				
MTM3-16	Exon 1 missing		Splicing error	
MTM20-12	Exon 2 missing		Splicing error	
MTM4-3	Exons 2 and 3 missing		Splicing error	
MTM19-9	Exons 2 and 3 missing		Splicing error	
MTM21-22	Exons 2 and 3 missing		Splicing error	
MTM13-3	Exon 3 missing		Splicing error	
MTM18-1	Exon 3 missing		Splicing error	
MTM13-2	Exon 5 missing		Genomic deletion	

[a] Exposed under Specific Aim 2 protocol; necropsy at 5 weeks after exposure.

[b] Numbering of the mouse *Hprt* gene according to Melton et al. 1984.

[c] The mutation in the nontranscribed strand is reported.

[d] The mutated base is in boldface and underlined. A series of boldfaced and underlined bases indicates a site of possible base deletion or insertion.

42 were considered to be independent mutations; and 7 mutations that were found more than once in clones from the same mouse were considered to be mutant siblings). Among independent base substitutions, 22 occurred at A•T base pairs (bp) and 12 occurred at G•C bp. Single-base insertions included one +G between bases 36 and 37 and another +G between bases 41 and 42. Single-base deletions included mutants with loss of one C, G, or A. One sizable deletion of 507 bp occurred between bases 43 and 549. After genomic DNA analysis, three independent exon exclusions were found to have been caused by splicing errors.

Of 201 *Hprt* mutants collected from BD-exposed (1250 ppm) male mice, 168 (approximately 84%) were successfully expanded for analysis by RT–PCR; 78 mutants yielded visible cDNA products. DNA sequencing showed that 11 cDNAs had wild-type *Hprt* sequences and 67 had base substitutions, frameshifts, complex mutations, or exon exclusions (Table 6). Of the 67 mutations, 65 were assumed to be independent mutations. Among independent base substitutions, 17 occurred at A•T bp and 21 occurred at G•C bp. Single-base deletions or insertions occurred at various positions. There were three complex mutations, including one G → TT at base 606, one AA → TT at bases 630 to 631, and an unusual mutation in which bases 487 to 571 were replaced by a segment of DNA including 50 bases in a noncoding region, exon 1, and part of exon 2 of the *Hprt* gene. In addition, there were eight mutants missing 1 or 2 exons in their cDNA products. After genomic DNA analysis, 7 of the exclusions were found to have been caused by splicing errors and 1 had a genomic DNA deletion of exon 5.

RT–PCR and cDNA Sequence Analysis of Mutants from Control and BD-Exposed Rats

A total of 195 *Hprt* mutants from control male rats were processed for colony expansion, and 156 (80%) of them were expanded to more than 5×10^5 cells. These 156 expanded mutant clones were analyzed by RT–PCR; 77 mutants produced cDNA products visible on 8% polyacrylamide gels. DNA sequencing showed that, of the 77 cDNA products, 28 had wild-type *Hprt* cDNA sequences and 49 had base substitutions, small insertions, or deletions (**Table 7**). Thirty-seven of the 49 were considered independent mutations. Among independent base substitutions, 15 occurred at A•T bp and 5 occurred at G•C bp. Insertions included one +CG between bases 114 and 115 and one +GGGCCC between bases 150 and 151. The 15 deletion mutations ranged in size from 21 to 586 bases.

Of 173 *Hprt* mutants collected from BD-exposed (1250 ppm) male rats, 160 were successfully expanded and analyzed by RT–PCR; 64 mutants produced visible cDNA products. DNA sequencing indicated that 22 cDNAs had wild-type *Hprt* sequences and 42 had base substitutions, frameshifts, deletions, or complex mutations (Table 7). Forty of the 42 mutations were considered independent mutations. Twenty independent base substitutions occurred at A•T bp

Table 7. RT–PCR and cDNA Sequence Analysis of *Hprt* Mutant Clones Isolated from Male Rats After a 2-Week Exposure to 0 or 1250 ppm BD (Specific Aim 5)[a]

Mutation Type and Animal Number	Position[b]	Mutation[c]	Sequence Context[d]	Amino Acid Change
Base Substitutions				
Control rats				
MRC2-6	49	T → C	GGT **T**AT GAC	Tyr → His
MRC18-16	124	A → T	CTG **A**TT ATG	Ile → Phe
MRC3-12	146	T → G	AGA C**T**T GCT	Leu → Arg
MRC4-3	146	T → G	AGA C**T**T GCT	Leu → Arg
MRC18-19	146	T → G	AGA C**T**T GCT	Leu → Arg
MRC18-12	215	A → G	GGC T**A**T AAG	Tyr → Cys
MRC3-1	228	T → C	TTT GC**T** GAC	Ala → Ala
MRC17-4	236	T → C	CTG C**T**G GAT	Leu → Pro
MRC17-9	236	T → C	CTG C**T**G GAT	Leu → Pro
MRC17-10	236	T → C	CTG C**T**G GAT	Leu → Pro
MRC8-5	279	T → C	TCC AT**T** CCT	Ile → Ile
MRC4-4	293	A → T	GTA G**A**T TTT	Asp → Val
MRC1-17	409	T → G	ATA A**T**T GAC	Ile → Ser
MRC1-5	431	A → C	ATG C**A**G ACT	Gln → Pro
MRC16-5	590	A → G	AAT G**A**G CAC	Glu → Gly
MRC16-6	590	A → G	AAT G**A**G CAC	Glu → Gly
MRC17-3	596	T → C	CAC T**T**C AGG	Phe → Ser
MRC6-3	605	T → G	GAT T**T**G AAT	Leu → Trp
MRC18-17	86	C → T	TAT G**C**T GAA	Ala → Val
MRC8-2	193	C → T	GCC **C**TC TGT	Leu → Phe
MRC8-1	202	C → G	GTG **C**TG AAG	Leu → Val
MRC3-8	204	G → A	GTG CT**G** AAG	Leu → Leu
MRC16-8	403	G → C	GAA **G**AT ATA	Asp → His

Table continues next page

[a] Exposed under Specific Aim 2 protocol; necropsy at 5 weeks after exposure.

[b] Numbering of the rat *Hprt* gene according to Jansen et al. 1992.

[c] The mutation in the nontranscribed strand is reported.

[d] The mutated base is in boldface and underlined. A series of boldfaced and underlined bases indicates a site of possible base deletion or insertion.

Table 7 (*Continued*). RT–PCR and cDNA Sequence Analysis of *Hprt* Mutant Clones Isolated from Male Rats After a 2-Week Exposure to 0 or 1250 ppm BD (Specific Aim 5)[a]

Mutation Type and Animal Number	Position[b]	Mutation[c]	Sequence Context[d]	Amino Acid Change
Base Substitutions (*Continued*)				
BD-exposed rats				
RTM16-16	49	T → C	GGT **T**AT GAC	Tyr → His
RTM25-13	49	T → C	GGT **T**AT GAC	Tyr → His
RTM28-14	72	A → C	TGC AT**A** CCT	Ile → Ile
RTM23-2	125	T → A	CTG A**T**T ATG	Ile → Asn
RTM21-16	146	T → A	AGA C**T**T GCT	Leu → His
RTM18-17	431	A → G	ATG C**A**G ACT	Gln → Arg
RTM25-4	437	T → C	ACT T**T**G CTT	Leu → Ser
RTM16-4	479	T → A	AAG G**T**T GCA	Val → Asp
RTM21-5	578	T → G	GCC C**T**T GAC	Leu → Arg
RTM16-12	603	T → G	AGG GA**T** TTG	Asp → Glu
RTM25-12	604	T → G	GAT **T**TG AAT	Leu → Val
RTM27-1	604	T → G	GAT **T**TG AAT	Leu → Val
RTM28-9	604	T → G	GAT **T**TG AAT	Leu → Val
RTM28-5	605	T → G	GAT T**T**G AAT	Leu → Trp
RTM29-1	605	T → A	GAT T**T**G AAT	Leu → Trp
RTM19-2	637	A → G	GGA **A**AA GCC	Lys → Glu
RTM21-15	637	A → G	GGA **A**AA GCC	Lys → Glu
RTM23-14	637	A → G	GGA **A**AA GCC	Lys → Glu
RTM28-3	637	A → G	GGA **A**AA GCC	Lys → Glu
RTM25-6	650	A → T	TAC A**A**A GCC	Lys → Ile
RTM16-14	43	C → G	GAA **C**CA GGT	Pro → Ala
RTM29-9	58	G → A	CAT **G**AT TTA	Asp → Asn
RTM27-6	211	G → A	GGG **G**GC TAT	Gly → Ser
RTM17-6	212	G → A	GGG G**G**C TAT	Gly → Asp
RTM24-20	580	G → A	CTT **G**AC TAT	Asp → Asn
Insertions				
Control rats				
MRC17-7	114–115	+ CT	CCT **CT** CAT	Frameshift
MRC17-8	114–115	+ CT	CCT **CT** CAT	Frameshift
MRC4-6	150–151	+ GGGCCC	GCT **G…C** CGA	In-frame insertion
BD-exposed rats				
RTM16-13	15–16	+ A	AGT **A** CCC	Frameshift
RTM20-4	553–555	+ A	CCA G**AA**C AAG	Frameshift
RTM19-1	562–563	+ C	TTT G**C**TT GTT	Frameshift
Deletions				
Control rats				
MR18-20-l	7–126	− 120 bp	TCG **ACC … ATT** ATG	In-frame deletion
MRC1-4	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift
MRC1-7	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift

Table continues next page

[a] Exposed under Specific Aim 2 protocol; necropsy at 5 weeks after exposure.

[b] Numbering of the rat *Hprt* gene according to Jansen et al. 1992.

[c] The mutation in the nontranscribed strand is reported.

[d] The mutated base is in boldface and underlined. A series of boldfaced and underlined bases indicates a site of possible base deletion or insertion.

Table 7 (*Continued*). RT–PCR and cDNA Sequence Analysis of *Hprt* Mutant Clones Isolated from Male Rats After a 2-Week Exposure to 0 or 1250 ppm BD (Specific Aim 5)[a]

Mutation Type and Animal Number	Position[b]	Mutation[c]	Sequence Context[d]	Amino Acid Change
Deletions (*Continued*)				
Control rats (*Continued*)				
MRC1-8	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift
MRC1-9	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift
MRC1-10	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift
MRC1-11	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift
MRC1-12	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift
MRC1-16	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift
MRC3-14-u	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift
MRC3-15-l	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift
MRC4-15	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift
MRC8-7	27–133	− 107 bp	GT**G ATT … A**GG	Frameshift
MRC3-14-l	28–402	− 375 bp	GTG **ATT … GAA** GAT	In-frame deletion
MRC7-2	30–615	− 586 bp	AT**T AGT …GTT** TGT	Frameshift
MRC18-8	135–318	− 184 bp	AG**G ACT … TGT** AAT	Frameshift
MRC3-11	319–384	− 66 bp	TGT **AAT … AAG** AAC	In-frame deletion
MRC6-4	321–386	− 66 bp	AA**T GAC … AA**C GTC	In-frame deletion
MRC18-20-u	321–386	− 66 bp	AA**T GAC … AA**C GTC	In-frame deletion
MRC8-8	403–456	− 54 bp	GAA **GAT … CAG** TAC	In-frame deletion
MRC18-15	486–532	− 47 bp	AG**C TTG … GAC T**TT	Frameshift
MRC15-10	533–553	− 21 bp	GAC T**TT … GA**C AAG	In-frame deletion
MRC17-16	533–553	− 21 bp	GAC T**TT … GA**C AAG	In-frame deletion
BD-exposed rats				
RTM28-6	51–669	− 619 bp	TA**T GAC … GCA** AGT	Frameshift
RTM25-9	135–318	− 184 bp	AG**G ACT … TGT** AAT	Frameshift
RTM28-4	216–654	− 439 bp	TA**T AAG … GCC** TAA	Frameshift
RTM18-16	256–441	− 186 bp	CTG **AAT … CTT** TCC	In-frame deletion
RTM26-11	318–384	− 67 bp	TG**T AAT … AAG** AAC	Frameshift
RTM16-11	385–402	− 18 bp	AAG **AAC …GAA** GAT	In-frame deletion
RTM19-9	385–485	− 101 bp	AAG **AAC … AG**C TTC	Frameshift
RTM19-12	385–485	− 101 bp	AAG **AAC … AG**C TTC	Frameshift
RTM19-16	385–485	− 101 bp	AAG **AAC … AG**C TTC	Frameshift
RTM25-17	403–609	− 207 bp	GAA **GAT … AAT** CAT	In-frame deletion
Complex Mutations				
Control rats				
No complex mutations observed				
BD-exposed rats				
RTM24-32	80	A → CTTATGC	AAT C**A**T TAT	His → ProTyrAla
RTM22-8	590–591	AG → CT	AAT G**AG** CAC	Glu → Ala
RTM23-5	599–600	GG → CT	TTC A**GG** GAT	Arg → Thr
RTM16-9	603–604	TT → GG	AGG GA**T T**TG	AspLeu → GluVal

[a] Exposed under Specific Aim 2 protocol; necropsy at 5 weeks after exposure.

[b] Numbering of the rat *Hprt* gene according to Jansen et al. 1992.

[c] The mutation in the nontranscribed strand is reported.

[d] The mutated base is in boldface and underlined. A series of boldfaced and underlined bases indicates a site of possible base deletion or insertion.

Table 8. Oligodeoxynucleotide Primers for Multiplex PCR Amplification of the Exon Regions of Mouse *Hprt* (Specific Aim 5)

Target Name	Product Length (bp)	Primer Name	Final Concentration of Primer (μM)	Primer Sequence (5′ → 3′)
Exon 1	320	MH1-5′	4.0	ATC AGG CCC ACC TAG TCA GA
		MH1-3′	4.0	CTC TGC TGG AGT CCC CTT G
Exon 2	112	MH2-5′	0.1	GCA GAT TAG CGA TGA TGA ACC
		MH2-3′	0.1	CCT GTC CAT AAT CAG TCC ATG A
Exon 3	379	MH3-5′	0.1	CCT CAT GCC CCA AAA TCT TA
		MH3-3′	0.1	CAC AGT AGC TCT TCA GTC TGA TAA AA
Exon 4	698	MH4-5′	0.9	ATC AGT CAA CGG GGG ACA
		MH4-3′	0.9	AGC ATT TGG GAG GCT GAA G
Exon 5	552	MH5-5′	0.5	TTT AAG GGC TCT GGT GGA TG
		MH5-3′	0.5	CAA CTC AGG CTA ACC CAG GA
Exon 6	485	MH6-5′	0.1	TTA AGG CCA CCA ACC TGA AC
		MH6-3′	0.1	TGG CAT ACA TAC CTT GCA ACC
Exon 7,8	262	MH7,8-5′	0.4	CTG GTG AAA AGG ACC TCT CG
		MH7,8-3′	0.4	CAA GGG CAT ATC CAA CAA CA
Exon 9	169	MH9-5′	0.1	AAC GAG AAT GTC TT TAT TTT ATA GCA
		MH9-3′	0.1	TTA CTA GGC AGA TGG CCA CA
K-*ras* Exon 2	191	RK2F	0.5	TTC TCA GGA CTC CTA CAG GAA A
		RK2R	0.5	CCC ACC TAT AAT GGT GAA TAT C

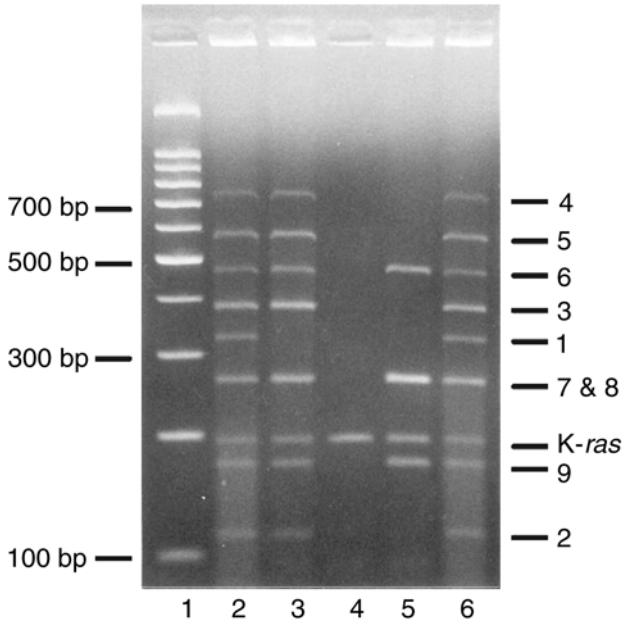

Figure 20. Multiplex PCR products amplified from several *Hprt* mutant clones isolated from BD-exposed male mice. The PCR products were separated on 2% agarose gel containing ethidium bromide and illustrated on a UV transilluminator. Labels on the left indicate fragment size in bp; products are identified by exon on the right. Lane 1, 100 bp DNA ladder; lanes 2 and 6, wild-type pattern of individual PCR products; lane 3, exon 1 product missing; lane 4, complete *Hprt* deletion (only exon 2 of K-*ras* was amplified); lane 5, products for exons 1 through 5 missing. (Specific Aim 5)

and 5 occurred at G•C bp. All frameshifts were single-base insertions. Deletions ranged in size from 18 to 619 bases, with most being larger than 100 bases. One deletion (bases 51 to 669) had only part of exon 1 remaining. There were 4 complex mutations, including one A → CTTATGC at base 80 and 3 mutants with tandem base substitutions (two-consecutive-base substitutions).

Multiplex PCR and Genomic DNA Analysis of Mutants from Control and BD-Exposed Mice

The sequences of the oligodeoxynucleotide primers, lengths of individual products, and final concentrations of each primer in the PCR reaction mixture for mouse *Hprt* are listed in Table 8. Several examples of multiplex PCR products amplified from *Hprt* mutant clones from male mice are shown in Figure 20.

The 57 mutants from control male mice that did not yield a visible cDNA product were analyzed by multiplex PCR of genomic DNA. Of these mutants, 24 had a wild-type pattern of PCR products on 2% agarose gels and the remaining 33 had one or more exons deleted (Table 9). Among the 33 mutants with deletions, 25 were considered independent; 10 occurred at the 5′ end of the *Hprt* gene, 7 occurred at the 3′ end, and 5 involved loss of internal exon(s). In cDNA from 3 clones, only K-*ras* exon 2 was suc-

Table 9. Multiplex PCR Analysis of Genomic DNA from *Hprt* Mutant T-Cell Clones Isolated from Male Mice and Rats After a 2-Week Exposure to 0 or 1250 ppm BD (Specific Aim 5)[a]

Type of Deletion	Control Mice		BD-Exposed Mice		Control Rats		BD-Exposed Rats	
	Total	Independent	Total	Independent	Total	Independent	Total	Independent
Exon 1	6	3	16	14	12	10	18	16
Exon 1–3	3	3	3	3	4	2	2	2
Exon 1–5	2	2	2	2	3	3	3	1
Exon 1–6	0	0	6	4	2	1	0	0
Exon 1–8	2	2	8	6	2	2	5	5
Exon 2–9	2	2	9	7	7	7	9	7
Exon 4–9	5	4	4	3	1	1	1	1
Exon 7–9	1	1	5	5	1	1	0	0
Exon 2	0	0	5	5	2	2	2	1
Exon 2–3	4	4	2	2	4	4	2	2
Exon 7–8	3	1	1	1	2	2	2	2
Exon 1–9	5	3	11	11	5	3	8	8
Total Number of Mutants								
With deletions	33	25	72	63	45	38	52	45
With no deletions	24	23	18	18	34	28	44	32
Screened	57	48	90	81	79	66	96	77

[a] Exposed under Specific Aim 2 protocol: necropsy at 5 weeks after exposure for mice and at 4 weeks for rats.

cessfully amplified; these were assumed to have complete deletions of the *Hprt* gene.

The 90 mutants from BD-exposed (1250 ppm) male mice that did not yield a visible cDNA product were analyzed by multiplex PCR. Of these, 18 mutants had wild-type PCR products and 72 had at least one exon deleted (Table 9). Among 63 independent mutations, the most common deletion (14 of 63) was loss of exon 1. Twenty-nine deletion mutations occurred at the 5′ end of *Hprt*, 15 occurred at the 3′ end, and 8 had internal exon deletions. In 11 mutants, only K-*ras* exon 2 was successfully amplified; these were assumed to have complete deletions of the *Hprt* gene.

Multiplex PCR and Genomic DNA Analysis of Mutants from Control and BD-Exposed Rats

The 79 mutants from control male rats that did not yield a visible cDNA product were processed for multiplex PCR analysis. Of these, 34 mutants had wild-type PCR products and the remaining 45 had at least one exon deleted. Thirty-eight of the 45 mutants were considered independent (Table 9). Eighteen deletion mutations occurred at the 5′ end of *Hprt*, 9 occurred at the 3′ end, and 8 had internal exon deletions. In 3 mutants, only K-*ras* exon 2 was successfully amplified; these were assumed to have complete deletions of the *Hprt* gene.

The 96 mutants from BD-exposed (1250 ppm) male rats that did not yield a visible cDNA product were analyzed by multiplex PCR. Of these, 44 mutants had wild-type PCR products and 52 mutants had at least one exon deleted (Table 9). Among 45 independent mutations, the most common type of deletion (16 of 45) was loss of exon 1. Twenty-four deletion mutations occurred at the 5′ end of the *Hprt* gene, 8 occurred at the 3′ end, and 5 had internal exon deletions. In 8 mutants, only K-*ras* exon 2 was successfully amplified; these were assumed to have complete deletions of the *Hprt* gene.

Comparison of Mutation Spectra in Control and BD-Exposed Male Mice

Table 10 summarizes the mutations in control and BD-exposed (1250 ppm) mice, as identified by RT–PCR and cDNA sequencing. The overall distribution of mutation types in control mice was different from that in BD-exposed mice ($P = 0.042$). The percentage of base substitutions was significantly lower in BD-exposed mice than in control mice ($P < 0.001$), mainly reflecting a marked decrease of A•T → G•C transitions in exposed mice ($P = 0.001$). In contrast, the percentage of G•C → C•G transversions was higher in BD-exposed mice than in controls, but the difference was only marginally significant ($P = 0.144$).

Table 10. RT–PCR and cDNA Sequence Analysis of *Hprt* Mutant T-Cell Clones Isolated from Male Mice and Rats After a 2-Week Exposure to 0 or 1250 ppm of BD (Specific Aim 5)[a]

Type of Mutation	Control Mice		BD-Exposed Mice		Control Rats		BD-Exposed Rats	
	Total	Independent	Total	Independent	Total	Independent	Total	Independent
Base substitutions	35	33 (79%)	40	38 (59%)[b]	23	20 (54%)	25	25 (62.5%)
G•C → A•T	7	7 (17%)	9	9 (14%)	3	3 (8%)	4	4 (10%)
A•T → G•C	15	14 (33%)	7	5 (8%)[b]	10	7 (19%)	8	8 (20%)
G•C → T•A	3	3 (7%)	5	5 (8%)	0	0	0	0
G•C → C•G	2	1 (2%)	7	7 (11%)	2	2 (5.5%)	1	1 (2.5%)
A•T → C•G	6	6 (14%)	7	7 (11%)	6	6 (16%)	7	7 (17.5%)
A•T → T•A	2	2 (5%)	5	5 (8%)	2	2 (5.5%)	5	5 (12.5%)
Frameshifts	8	5 (12%)	16	16 (25%)	0	0	3	3 (7.5%)
+ 1	3	2 (5%)	7	7 (11%)	0	0	3	3 (7.5%)
−1	5	3 (7%)	9	9 (14%)	0	0	0	0
Insertions	0	0	0	0	3	2 (5.5%)	0	0
Deletions	1	1 (2%)	0	0	23	15 (40.5%)	10	8 (20%)
Complex mutations	0	0	3	3 (5%)	0	0	4	4 (10%)
Exon exclusions	5	3 (7%)	8	8 (12%)	0	0	0	0
Total	49	42 (100%)	67	65 (100%)	49	37 (100%)	42	40 (100%)[b]

[a] Exposed under Specific Aim 2 protocol: necropsy at 5 weeks after exposure for mice and at 4 weeks for rats. Percentage of total given in parentheses.

[b] $P < 0.05$ in testing the difference between control and BD-exposed mice or rats.

Frameshifts constituted a substantial portion of *Hprt* mutations in BD-exposed mice ($P = 0.01$), although the increase over background (25% vs. 12%) was not statistically significant ($P = 0.137$).

Table 9 summarizes the deletion mutations in control and BD-exposed mice, as identified by multiplex PCR. Statistical analysis of the spectra data for genomic DNA showed that deletions in general (i.e., 5′ partial deletions, 3′ partial deletions, internal deletions, and complete gene deletions) occurred significantly more frequently in BD-exposed mice than in control mice ($P = 0.031$), although no individual subcategory of deletions occurred significantly more frequently.

Table 11 presents the results of RT–PCR and cDNA analysis and of multiplex PCR of genomic DNA from *Hprt* mutants isolated from control and BD-exposed mice. The table summarizes the total mutations observed, the total independent mutations, and the calculated mutant fractions for each class and type of mutation found. (Table 12, discussed below, presents parallel data for control and BD-exposed rats.) After combining the data from RT–PCR and cDNA sequencing and from multiplex PCR analyses, the overall distribution of mutation types in control mice versus BD-exposed mice fell just short ($P = 0.061$) of showing a statistical difference. However, the induced mutant fractions (BD mutant fraction minus spontaneous mutant fraction) do provide evidence of a chemically induced mutagenic response. Assuming the mutant counts were distributed as Poisson variates, comparisons of the mutant fractions for each type of mutation in BD-exposed mice with those in control mice demonstrated that BD exposure significantly increased the frequencies of all types of base substitutions (except A•T → G•C transitions), both +1 and −1 frameshifts, deletion mutations in general, and splicing errors (Table 11). The increase in BD-induced deletion mutations consisted chiefly of statistically significant increases in 5′ partial deletions, 3′ partial deletions, and complete *Hprt* deletions.

Comparison of Mutation Spectra in Control and BD-Exposed Male Rats

Using the RT–PCR and cDNA sequencing data alone or in combination with the multiplex PCR data, statistical analysis of the overall distribution of mutation types in control versus BD-exposed (1250 ppm) rats did not show a significant difference. After combining the cDNA and genomic DNA mutation data (Table 12), statistical analysis of the mutant fractions for individual types of mutations among control versus BD-exposed rats showed that BD exposure produced statistically significant ($P < 0.05$)

Table 11. Mutations in *Hprt* Mutant Fractions in T Cells of Male Mice After a 2-Week Exposure to 0 or 1250 ppm BD (Specific Aim 5)[a,b]

	Control Mice			BD-Exposed Mice			
Type of Mutation	Mutants	Independent	Mutant Fraction	Mutants	Independent	Mutant Fraction (P value[c])	BD-Induced Mutant Fraction[d]
Base substitutions	35 (43%)	33 (49%)	7.29	40 (29%)	38 (30%)	27.31 (< 0.001)	20.02 (26%)
G•C → A•T	7 (9%)	7 (10%)	1.55	9 (6%)	9 (7%)	6.47 (0.005)	4.93 (6%)
A•T → G•C	15 (18%)	14 (21%)	3.09	7 (5%)	5 (4%)	3.59 (0.501)	0.50 (1%)
G•C → T•A	3 (4%)	3 (4%)	0.66	5 (4%)	5 (4%)	3.59 (0.021)	2.93 (4%)
G•C → C•G	2 (2%)	1 (1%)	0.22	7 (5%)	7 (5%)	5.03 (< 0.001)	4.81 (6%)
A•T → C•G	6 (7%)	6 (9%)	1.33	7 (5%)	7 (5%)	5.03 (0.017)	3.70 (5%)
A•T → T•A	2 (2%)	2 (3%)	0.44	5 (4%)	5 (4%)	3.59 (0.010)	3.15 (4%)
Frameshifts	8 (10%)	5 (7%)	1.10	16 (12%)	16 (13%)	11.50 (< 0.001)	10.40 (13%)
+ 1	3 (4%)	2 (3%)	0.44	7 (5%)	7 (5%)	5.03 (< 0.001)	4.59 (6%)
− 1	5 (6%)	3 (4%)	0.66	9 (7%)	9 (6%)	6.44 (< 0.001)	5.81 (8%)
Deletions	34 (41%)	26 (39%)	5.74	73 (53%)	64 (50%)	46.00 (< 0.001)	40.26 (52%)
5′ Partial	13 (16%)	10 (15%)	2.21	35 (25%)	29 (23%)	20.84 (< 0.001)	18.63(24%)
3′ Partial	8 (10%)	7 (11%)	1.54	18 (13%)	15 (12%)	10.78 (< 0.001)	9.23 (12%)
Internal	8 (10%)	6 (9%)	1.33	9 (6%)	9 (7%)	6.47 (0.003)	5.14 (7%)
Complete	5 (6%)	3 (4%)	0.66	11 (8%)	11 (9%)	7.91 (< 0.001)	7.24 (10%)
Splicing errors	5 (6%)	3 (4%)	0.66	7 (5%)	7 (4%)	5.03 (0.002)	4.37 (6%)
Complex mutations	0	0	0	3 (2%)	3 (2%)	2.16 (0.013)	2.16 (3%)
Total	82 (100%)	67 (100%)	14.80	139 (100%)	128 (100%)	92.00	77.20 (100%)

[a] Exposed under Specific Aim 2 protocol; necropsy at 5 weeks after exposure. Percentage of total given in parentheses.

[b] Mutant fraction = (observed Mf $\times 10^{-7}$) $\times$ (percent of type of mutation). Calculations used combined results of RT–PCR/cDNA and multiplex PCR analyses.

[c] P values are shown for significant differences between mutant fractions for individual mutation types in BD-exposed mice compared with controls.

[d] BD-induced mutant fraction = observed mutant fraction for BD-exposed animals minus the mutant fraction for control animals.

Table 12. Mutations in *Hprt* Mutant Fractions in T Cells of Male Rats After a 2-Week Exposure to 0 or 1250 ppm BD (Specific Aim 5)[a,b]

	Control Rats			BD-Exposed Rats			BD-Induced Mutant Fraction[d]	
Type of Mutation	Total	Independent	Mutant Fraction	Total	Independent	Mutant Fraction (P value[c])	Rats	Mice[e]
Base substitutions	23 (25%)	20 (27%)	8.18	25 (27%)	25 (29%)	23.76 (<0.001)	15.58 (31%)	20.02 (26%)
G•C → A•T	3 (3%)	3 (4%)	1.23	4 (4%)	4 (5%)	3.80 (0.127)	2.57 (5%)	4.93 (6%)
A•T → G•C	10 (11%)	7 (9%)	2.87	8 (9%)	8 (9%)	7.60 (0.051)	4.74 (9%)	0.50 (1%)
G•C → T•A	0	0	0	0	0	0	0	2.93 (4%)
G•C → C•G	2 (2%)	2 (3%)	0.82	1 (1%)	1 (1%)	0.95 (0.132)	0.13 (<1%)	4.81 (6%)
A•T → C•G	6 (6%)	6 (8%)	2.46	7 (8%)	7 (8%)	6.65 (0.063)	4.20 (8%)	3.70 (5%)
A•T → T•A	2 (2%)	2 (3%)	0.82	5 (5%)	5 (6%)	4.75 (0.029)	3.93 (8%)	3.15 (4%)
Frameshifts								10.40 (13%)
+ 1	0	0	0	3 (3%)	3 (4%)	2.85 (0.027)	2.85 (6%)	4.59 (6%)
−1	0	0	0	0	0	0	0	5.81 (8%)
Deletions	66 (70%)	53 (71%)	21.69	62 (66%)	53 (62%)	50.38 (<0.001)	28.67 (57%)	40.26 (52%)
5′ Partial	21 (22%)	18 (24%)	7.37	28 (30%)	24 (28%)	22.81 (<0.001)	15.45 (31%)	18.63 (24%)
3′ Partial	9 (10%)	9 (12%)	3.68	10 (11%)	8 (9%)	7.60 (0.106)	3.92 (8%)	9.24 (12%)
Internal	31 (33%)	23 (31%)	9.41	16 (17%)	13 (15%)	12.36 (0.268)	2.94 (6%)	5.14 (7%)
Complete	5 (5%)	3 (4%)	1.23	8 (9%)	8 (9%)	7.60 (0.004)	6.37 (13%)	7.24 (10%)
Insertions	3 (3%)	2 (3%)	0.82	0	0	0	−0.082 (−2%)	0
Splicing errors	0	0	0	0	0	0	0	4.37 (6%)
Complex mutations	0	0	0	4 (4%)	4 (5%)	3.80 (0.008)	3.80 (8%)	2.16 (3%)
Total	94 (100%)	75 (100%)	30.7	94 (100%)	85 (100%)	80.8	50.10 (100%)	77.20 (100%)

[a] Exposed under Specific Aim 2 protocol; necropsy at 4 weeks after exposure. Percentage of total given in parentheses.

[b] Mutant fraction = (observed Mf $\times$ 10^{-7}) $\times$ (percent of type of mutation). Calculations used combined results of RT–PCR/cDNA and multiplex PCR analyses.

[c] P values are shown for significant differences between mutant fractions for individual mutation types in BD-exposed rats compared wirh controls.

[d] BD-induced mutant fraction = observed mutant fraction for BD-exposed animals minus the mutant fraction for control animals.

[e] Data for percent contribution of BD-induced mutant fractions for different mutation types for mice are taken from Table 11.

increases in base substitutions overall, with a significant increase in A•T → T•A transversions ($P = 0.029$) and marginally significant increases in A•T → G•C transitions ($P = 0.051$) and A•T → C•G transversions ($P = 0.063$). In addition, there was evidence of significant increases in +1 frameshifts, deletion mutations in general, and complex mutations ($P = 0.027$ to < 0.001). The increase in BD-induced deletion mutations consisted chiefly of significant increases in 5′ partial deletions and complete *Hprt* deletions ($P \leq 0.004$). The last two columns in Table 12 provide instructive comparisons of the relative extent of the increases in individual classes and types of BD-induced mutations in male rats versus mice under the same experimental exposure conditions.

Comparison of Mutation Spectra in Control Mice and Control Rats

Comparison of the distribution data for 67 independent mutations in control mice (Table 11) to the data for 75 independent mutations in control rats (Table 12) showed that the proportion of base substitutions in mice was significantly higher than that in rats (43% vs. 23%, $P < 0.001$) and that this difference consisted chiefly of increases in G•C → A•T transitions, A•T → G•C transitions, and G•C → T•A transversions ($P = 0.001$, 0.001, and 0.01, respectively). The increases in these base substitutions in mice were accompanied by a significant decrease in the proportion of deletion mutations in mice compared with rats (39% vs. 71%; $P < 0.001$). As pointed out above, most of these internal deletions in the rat were of a size and type detected in *Hprt* cDNA (15 of 23). Additional significant findings included decreases in the proportions of +1 frameshifts ($P = 0.05$), −1 frameshifts ($P = 0.01$), and splicing errors ($P = 0.01$) in control rats compared with control mice.

Among the mutations that readily lend themselves to direct comparisons, there was remarkably little overlap in the mutation-spectra data between control and BD-exposed mice, control and BD-exposed rats, control mice and control rats, and BD-exposed mice and rats in terms of the sites of base substitutions or single-base deletions or insertions. Overlap was limited to the occurrence of (1) the same mutation at base 313 (T → C) in control and BD-exposed mice; (2) the same mutation at base 49 (T → C) and base 605 (T → G) in control and BD-exposed rats; (3) different mutations at base 146 and base 431 of control and BD-exposed rats; (4) the same mutation at base 86 (C → T) and different mutations at base 605 in control mice and rats; (5) the same mutation at base 211 (G → A) and base 604 (T → G) in BD-exposed mice and rats; and (6) a different mutation at base 58 of BD-exposed mice and rats.

DISCUSSION AND CONCLUSIONS

In a series of investigations sponsored under HEI's air toxics research program, our team has studied species-specific patterns in DNA damage and mutagenic responses in rodents exposed to BD and its metabolites. Our goals were to characterize the early effects of BD exposure on mice and rats in order to explain the higher sensitivity of mice to BD-induced carcinogenesis and thus to help discover which animal is the more useful surrogate for predicting health effects in people exposed to BD.

In our first investigation, we conducted a series of studies to (1) determine species differences in the mutagenicity of BD in exposed mice and rats; (2) estimate the relative contribution of BDO and BDO_2 (as represented by the racemic mixtures) to the mutagenic effects of BD in each species; (3) characterize in a limited manner the small-scale mutations induced in mice and rats exposed to BD versus (±)-BDO_2; and (4) improve culture techniques for the large-scale expansion of rodent T-cell clones for future identification of BD-induced *Hprt* mutations.

In our current investigation, we conducted a series of studies that demonstrated that (1) the exposure–response relations between BD exposures (to 3 ppm and greater), alkylation of hemoglobin and DNA, and induction of *Hprt* mutations are in agreement with predictions from mutagenic-potency estimates for mice (see Tables 4 and 5); (2) the difference between the in vivo mutagenic potencies of *meso*-BDO_2 and (±)-BDO_2 is much less consequential than the differences in mutagenic potency among the three major epoxy metabolites (BDO, BDO_2, and BDO-diol) without regard to stereoisomerism; (3) the spectra of BD-induced mutations, established by identification of both large- and small-scale mutations, are more varied and indicate more deletion mutations than previously shown in mice or envisioned in rats; and (4) these exposure–response relations, mutation-spectra data, and mutagenic potency estimates for BD-diol–derived metabolites (chiefly BDO-diol), along with those previously obtained for BD, BDO, and BDO_2, can be used to draw conclusions about the metabolite(s) responsible for the differences in induced mutations found in mice compared with rats over a range of BD exposure levels.

Additional research in the current project demonstrated that, although the shapes of the *Hprt* mutant splenic T-cell manifestation curves were different for two distinct age groups of BD-exposed mice, the ultimate mutagenic burden was the same for both age groups. These results indicate that the diverse results obtained in earlier *Hprt* mutation studies in BD-exposed mice and rats primarily reflect age-related differences in the trafficking of T cells and not age-related differences in the biotransformation of BD.

The findings reported here greatly expand our current knowledge of species differences in mutagenic responses to BD, fill significant gaps in the exposure–response paradigm for BD exposure, and substantially improve the scientific basis of BD risk assessment.

BIOLOGIC FACTORS THAT AFFECT MEASURING *Hprt* M*f*S AND QUANTIFYING MUTAGENIC RESPONSES TO BD EXPOSURE IN MICE AND RATS

Over the course of our investigations, we recognized that after rodents are exposed to a mutagen, numerous factors can affect the in vivo perturbation of T cells and the subsequent quantification of *Hprt* M*f*s. Studies with the direct alkylating chemical ethylnitrosourea, for example, demonstrated that species, animal age, chemical dose, dose rate and schedule, source of lymphocytes (thymus, spleen, or lymph nodes), and time elapsed after exposure all affect the measured M*f* or observed mutagenic response (Jones et al. 1987a; Aidoo et al. 1993; Meng et al. 1998a; Walker et al. 1999; Walker and Meng 2000). Previous studies of mice exposed to ethylnitrosourea showed that induced mutation patterns are organ-specific, with early increases in M*f* seen in thymic T cells followed by later increases in splenic T cells (Jones et al. 1987a; Walker et al. 1999; Judice 2001). In experiments investigating the age-related appearance and disappearance of ethylnitrosourea-induced mutant T cells in male mice, it was observed that the time needed to reach peak M*f* values in thymus was fairly uniform regardless of the animal's age at exposure, whereas the time needed to reach peak M*f* values in spleen was roughly proportional to the animal's age at exposure (Jones et al. 1987a; Walker et al. 1999). These data indicated that age-related differences in the appearance and disappearance of mutant T cells in spleen (and peripheral lymphoid tissues) are largely determined by the fixation of mutations during T-cell maturation and age-related trafficking of mutant T cells through the thymus.

To address these factors, we developed a novel approach for estimating total mutagenic burden by integrating the area under the *Hprt* mutant T-cell manifestation curves. This would allow us to compare different rodent species and the effects of exposure to BD and its metabolites under different exposure protocols. A description and validation of this approach have been presented elsewhere (Meng et al. 1998a; Walker and Meng 2000).

Quantifying mutagenic potency as the change in frequency of *Hprt* mutant splenic T cells over time after exposure proved to be the best way to derive both species- and age-related (when mice and rats are the same age) comparisons of total mutagenic burden, in the absence of quantitative data on critical parameters (e.g., cell-cycle rates, in vivo half-life values, T-cell trafficking rates, and T-cell clonality) that might influence M*f*s at any particular time after a given exposure to an alkylating agent.

Effects of T-Cell CE and Clonality

The positive results of earlier exposure–response studies of *Hprt* M*f*s in splenic T cells of female mice exposed to BD at levels as low as 20 ppm suggested that significant mutagenic responses might be detectable at even lower exposure levels (Meng et al. 1998a, 1999a; Walker and Meng 2000) that would approach the U.S. Occupational Safety and Health Administration's occupational exposure threshold for BD. Indeed, initial statistical analysis of CE and M*f* data (using the Mann-Whitney Rank Sum test) in the current investigation indicated that exposure to as little as 3 ppm BD induced a small but significant mutagenic response in mice. However, in order to establish whether low levels of BD induce detectable mutagenicity at the *Hprt* locus of mouse T cells, the potential impact of variables that affect the background frequency of *Hprt* mutant T cells in rodents and humans needed to be considered. In healthy, unexposed adult humans, the three factors that most affect background M*f*s are age, CE, and the occurrence of clonality among *Hprt* mutant T cells (Albertini 1985; Cole et al. 1988; Tates et al. 1991). With the exception of our age-related BD experiments (Specific Aim 2), variation in age was not a concern in the current project because mice and rats of the same age were used. Also, the narrow range of reasonably good CEs obtained for control mice and mice exposed to 3 ppm BD (Specific Aim 1) suggested that the "lower" CE values in the data set had no effect on M*f* values (Table 2). As discussed elsewhere (Meng et al. 2001), this lack of impact of CE on M*f* values in mice in low-dose BD experiments is reasonable, because the number of covariates is smaller (i.e., the mice were all the same age, were fed the same diet, were not exposed to tobacco smoke, and were syngeneic) than in studies of adult human populations (Tates et al. 1991, 1994; Meng et al. 1998b).

The occurrence of clonality among *Hprt* mutant T cells can distort the relation between mutation events and the resulting frequency of mutant cells in humans and rodents (O'Neill et al. 1994; Walker et al. 1999; Judice 2001). However, this phenomenon is of much less concern in children and rodents because of thymic activity. The contribution of *Hprt* mutant T-cell siblings to in vivo M*f*s in peripheral blood and lymphoid tissue can be assessed by examining mutant clones for T-cell–receptor gene rearrangements. The interpretation of such assessments is relatively straightforward in adult humans, because thymic activity ceases during the second decade of life. Clonality, or the

occurrence of mutant siblings with the same *Hprt* mutation and the same T-cell–receptor gene arrangements, is a minor problem in most adults in the absence of a mutator phenotype (O'Neill et al. 1994). O'Neill and colleagues have recommended that clonal amplification of *Hprt* T-cell mutants, despite their biologic relevance, need not be corrected in estimating mean M*f*s in populations of adult humans, provided that outliers are recognized.

Current knowledge of T-cell renewal and trafficking in children and rodents suggests that occasional duplicate mutations among a group of *Hprt* mutant clones isolated from the cord blood of a single infant or from the spleen of a single mouse or rat are more likely to originate from mutations initially induced (spontaneously or by chemical exposure) in mitotically active prethymocytes or thymocytes. These duplicate mutations may represent either mutant siblings or independent mutations (Crippen and Jones 1989; Finette et al. 1994, 1996, 1998; Walker et al. 1999; Walker and Meng 2000; O'Neill et al. 2001). These phenomena were recently investigated in mice by analyzing *Hprt* mutant T-cell isolates for two molecular markers of clonality, *Hprt* and T-cell–receptor gene changes (Judice 2001). Analyses of T-cell mutants isolated from thymus and spleen of male mice after exposure to ethylnitrosourea produced definitive evidence of (1) mutagenesis occurring primarily in pre–T cells and thymus, (2) the flow of *Hprt* mutant T cells from thymus to spleen (and peripheral blood), and (3) the subsequent dilution of splenic *Hprt* mutants by sustained T-cell maturation and selective pressures. These findings show that, as in the case of children (Finette et al. 1994, 1996, 1998), *Hprt* mutations in rodents are "fixed" predominantly during T-cell maturation and that clonality is *Hprt*-defined (Judice 2001). The occurrence of clonality is an integral and inseparable part of the mutagenic response that is measured by the T-cell cloning assay in children and rodents. Thus, all *Hprt* mutant T cells, including mutant siblings, should be counted in determining the background M*f*s and the mutagenic response of children and rodents to exposure to DNA-damaging agents. Duplicate *Hprt* mutations (as determined by mutation-spectra and T cell–receptor gene analysis) in children and rodents should not be routinely corrected for clonality, except in those few cases where the M*f* value represents an outlier shown to be the consequence of a "dominant" clonal expansion. Figure 5 shows that there were no outliers in M*f* values in either control mice or mice exposed to 3 ppm BD. In this case, then, it would be inappropriate to adjust the measured *Hprt* M*f*s for clonality.

Age- and Sex-Related Differences in Mutagenic Response

The underlying goals of experiments to examine possible age- and sex-related differences in the mutagenic response of BD-exposed mice (Specific Aim 2) were to (1) identify the principal factors that affect the occurrence of genetic change at *Hprt* of T cells in mice of different ages or sexes, and (2) facilitate the design and interpretation of future investigations examining the mutagenic effects of BD and other alkylating agents.

Among the variables that complicate comparisons between the results of earlier *Hprt* mutation studies of BD and its metabolites in rodents (Cochrane and Skopek 1994b; Tates et al. 1994, 1998; Meng et al. 1998a, 1999a; Walker and Meng 2000), Tates and colleagues (1998) emphasized that the differing mutagenic responses in these studies might be caused primarily by age-specific differences in (1) thymus activity and trafficking of T cells and (2) the biotransformation of BD. In our ethylnitrosourea studies (Walker et al. 1999), the mutagenic potency of the exposure regimen (40 mg ethylnitrosourea/kg by intraperitoneal injection) was significantly greater (1.6-fold) in mice exposed as preweanlings (12 days old) than in those exposed as young adults (8 weeks old). However, Tates and colleagues (1998) overlooked the fact that the estimated mutagenic potencies for this dose of ethylnitrosourea (based on mutant T-cell manifestation curves for exposed versus control animals) were the same for young adult mice and weanlings (3 weeks old). Therefore, in mice (and rats) exposed to direct alkylating agents at 3 weeks of age and older, the shapes of the mutant T-cell manifestation curves will differ but the area under the curves (or overall mutagenic response) should be similar (Walker et al. 1999). Apart from age-related differences in T-cell kinetics, the important question raised by Tates and colleagues (1998) was "Do age-specific differences in drug transformation contribute to age-specific differences in the mutagenic potency of (an indirect mutagen like) BD?"

Age-related differences in DNA repair have also been proposed as a potential factor in age-related differences in chemically induced mutations and cancer. For example, age-related differences in the burden of tumors induced by ethylnitrosourea have been attributed, in part, to differences in age-related activities of O^6-methylguanine-DNA-methyltransferase and 3-methyladenine-DNA-glycosylase (Washington et al. 1988, 1989). These DNA-repair enzymes were consistently lower in various tissues of preweanling mice (9 days old) compared with young adults (7 to 8 weeks old; Washington et al. 1988, 1989). In contrast, there is a paucity of data on the promutagenic adducts of BD metabolites and the mechanisms involved in their repair.

The working hypothesis of our experiment on the impact of age differences was that the enzymes involved in the activation and detoxification of BD (e.g., P450 monooxygenases, epoxide hydrolases [EHs], and glutathione transferases [Himmelstein et al. 1994, 1995, 1996; Thorton-Manning et al. 1995a,b, 1996]) were not sufficiently different between 4- to 5-week-old mice (consistently used in our previous BD-related studies; Meng et al. 1998a, 1999a,b, 2000; Walker and Meng 2000) and adult mice (e.g., 8 weeks old) to affect the mutagenic potency of a given exposure regimen (e.g., 2 weeks of exposure to 1250 ppm BD). The resulting mutagenicity data for BD-exposed 4- to 5-week-old and 8-week-old female mice, characterized by mutant manifestation curves of different shapes (Figure 7) but similar mutagenic-potency estimates (Table 4), strongly support our premise that mutagenic responses induced by BD and its metabolites in 4- to 5-week-old rodents are representative of those induced in older (adult) animals. These findings further strengthen our contention that the ability to detect and estimate the potency of a weakly mutagenic chemical can be increased by using 6-week-old rodents that are sexually mature, have full metabolic capabilities, and have thymuses with 50% to 100% of their maximal number of cells (Walker et al. 1999). Compared with the use of rodents at 3 months of age (the approximate age group used by Tates and colleagues [1994, 1998]), the use of younger adult animals compresses the time frame of the mutant T-cell manifestation curve for spleen, increases the maximal M*f* detected, and reduces the number of time points and animals needed to demonstrate the change in *Hprt* M*f* over time after exposure (Walker et al. 1999).

Another experiment was performed because reports of sex-related differences in metabolic activation and detoxification of BD in mice and rats (Himmelstein et al. 1994, 1995, 1996; Thorton-Manning et al. 1995a,b, 1996) have suggested that this phenomenon might ultimately lead to sex-related differences in mutagenesis. The results of our experiments on sex-related differences, along with our earlier (and current) experiments on species-related differences, clearly demonstrate that the overall order in degrees of mutagenic susceptibility in BD-exposed rodents is female mice > male mice > female rats > male rats. These data are reassuring in that they fit current knowledge of sex- and species-related differences in BD metabolism and carcinogenic susceptibility (Huff et al. 1985; Owen et al. 1987; Melnick et al. 1990; Melnick and Huff 1992; Himmelstein et al. 1994, 1995, 1996; Thorton-Manning et al. 1995a,b, 1996; Walker and Meng 2000). However, it is surprising that systematic measurements of BD metabolites have been made in the blood and tissues of female rats, male rats, and male mice, but not of female mice (except for the limited measurements reported here), even though the female mouse is the most sensitive to BD-induced mutagenesis and carcinogenesis. Our findings of greater mutagenic responses in both female mice and rats also raise an important question as to whether female humans are more susceptible than males to the potentially toxic effects of BD exposure in the environment or workplace.

The results of these age- and sex-related experiments help explain part of the differing outcomes in earlier *Hprt* mutagenicity studies of BD-exposed rodents. As one example, Tates and colleagues (1994, 1998) used male mice and rats in their studies and tried to compare their results with our studies in female rodents, unaware of the sex difference in mutagenic susceptibility. Moreover, in our earlier BD mutagenicity studies, we would probably have obtained largely negative results if we had used male mice and rats in our first assessments of species differences because knowledge of how best to perform the experiments on the less sensitive sex was unavailable (Meng et al. 1998a; Walker and Meng 2000). Thus, data from the current investigation support the predictive value of *Hprt* data derived from the relatively young animals selected for our earlier investigations. This body of work further demonstrates the need for (1) greater uniformity in the design of chemical-specific mutagenicity studies in order to facilitate cross-study comparisons among laboratories that use the rodent T-cell *Hprt* mutation assay, and (2) the inclusion of both sexes when significant differences in their responses to chemical exposure have been established.

COMPARISONS OF MUTAGENIC POTENCIES OF BD, BDO, (±)-BDO_2, *meso*-BDO_2, AND BD-DIOL EXPOSURES IN MICE AND RATS

In our earlier HEI investigation (Walker and Meng 2000), we conducted experiments on the relative contribution of the racemic mixtures of BDO and BDO_2 to the total mutagenicity of BD by exposing mice and rats to selected levels of BD and these epoxy intermediates. We also compared the mutagenic potency of each compound when comparable blood concentrations of the metabolites were attained. In the current investigation, we extended our work to make analogous mutagenic-potency estimates for mice and rats exposed to *meso*-BDO_2 or BD-diol (as a source of BDO-diol), with the ultimate goal of obtaining sufficient information to draw conclusions about the degree to which different epoxy intermediates and the stereoisomers of BDO_2 induce mutations in BD-exposed mice and rats.

In our inhalation studies of *meso*-BDO_2, repeated (4-week) exposures to this stereospecific metabolite caused a significant increase in *Hprt* M*f*s in the splenic T cells of both rats and mice. As in the case of rodents exposed to (±)-BDO_2, the mutagenic effects were greater in rats than in mice (Figures 9 and 10). These effects may be related to at least two factors. First, based on in vitro studies, the rate of hepatic clearance of BDO_2 by hydrolysis and glutathione conjugation in rat tissue is 2.3-fold lower than in mouse tissue (Boogaard and Bond 1996; Boogaard et al. 1996), which could lead to higher blood and tissue concentrations of BDO_2 in rats than in mice exposed to the same concentrations of BDO_2. Second, rat T cells appear to tolerate the cytotoxic effects of BDO_2 better than mouse T cells (Henderson et al. 1997), which might lead to the detection of higher *Hprt* M*f*s in rats.

Several research groups have evaluated the mutagenicity of (±)-BDO_2 in mice and rats (Cochrane and Skopek 1994b; Tates et al. 1998; Meng et al. 1999b; Recio et al. 2001), but this report presents the first demonstration that *meso*-BDO_2 exposures cause significant mutagenic effects in both sexes of mice and rats (Figures 9 and 10). Moreover, the shape of the *Hprt* mutant splenic T-cell manifestation curves in female mice and rats exposed to *meso*-BDO_2 (Figure 9) resembled those obtained from female mice and rats exposed to the same level of (±)-BDO_2 (see Figure 11 in Walker and Meng 2000). A cursory comparison of the curves suggests differences in the response pattern, because peak M*f*s were reached at two different times after exposure to the two different stereoisomers. However, the two different times are related to T-cell kinetics and the fact that the animals were exposed to *meso*-BDO_2 for only 3 weeks and to (±)-BDO_2 for 4 weeks (Meng et al. 1999b; Walker and Meng 2000). (The rodents, 4 to 5 weeks old at start, aged another week during the 4th week of exposure, with the result that, because of the age-related trafficking of T cells, an additional 1 to 2 weeks elapsed after exposure before peak M*f*s were reached.)

Comparisons of the mutagenic-potency estimates for exposures of female rats to 2 ppm (±)-BDO_2 for 4 weeks or 2 ppm *meso*-BDO_2 for 3 weeks (6 hours/day, 5 days/week) demonstrated that these exposures caused equivalent mutagenic effects. In female mice, the same exposure protocols produced a nearly threefold greater mutagenic response to the exposure to (±)-BDO_2. However, this difference in response to the two stereoisomers would probably decrease after an additional week of exposure (4 weeks total) to *meso*-BDO_2. Although exact comparisons of the mutagenic potencies of (±)-BDO_2 and *meso*-BDO_2 cannot be made because of the differences in exposure durations, these M*f* data are sufficient to support the conclusion that there is little difference in the in vivo mutagenic potencies of the stereochemical forms of BDO_2 in rodents.

This conclusion is also supported by a preliminary report on the mutagenicity of *meso*-BDO_2, *R*,*R*-BDO_2 (D), and *S*,*S*-BDO_2 (L) (Figure 2) at the *TK* and *HPRT* loci in human lymphoblastoid cells, in which all three of these stereoisomers produced higher frequencies of mutations in *TK* (representing chiefly large-scale genetic changes) than in *HPRT*. But there were no significant differences in exposure–related mutagenic responses to the individual stereoisomers themselves (Meng et al. 2007). These data indicate that the small differences in the mutagenic potencies of the stereoisomers of BDO_2 appear to be far less consequential than the much larger differences in the mutagenic potencies of BDO_2 (without regard to stereochemistry), BDO, and BDO-diol (which have an in vitro mutagenic-potency ratio of 200:2:1, respectively [Cochrane and Skopek 1994a]). Thus, the stereochemistry of these epoxy compounds probably does not play a significant role in the mutagenicity and carcinogenicity of BD.

An original goal of this project was to determine the relative contribution of BDO-diol to BD-induced mutagenicity in rodents by exposing mice and rats directly to a well-characterized mixture of the stereoisomers of BDO-diol. However, upon further consideration we felt that more definitive results could be obtained by exposing mice and rats directly to BD-diol at levels that produce blood concentrations of BD-diol equivalent to those found after exposure of mice to selected levels of BD. BD-diol is not a DNA-reactive agent; it is the proximate intermediate in the elimination pathways that lead to BDO-diol and HMVK. Inhalation exposure experiments in mice and rats exposed to BD and BD-diol demonstrated that (1) BD-diol accumulated in blood at high concentrations in a sublinear manner at BD exposure levels higher than 200 ppm; (2) BD-diol was excreted in urine in minor amounts (compared with M1 and M2) after BD exposure; and (3) direct exposure to BD-diol caused significant mutagenic effects in both sexes of mice and rats. Small but significant mutagenic responses were found in mice, but not rats, after 4 weeks of exposure (6 hours/day, 5 days/week) to 6 ppm BD-diol. Furthermore, BD-diol was detected in the plasma of mice and rats exposed to 6 ppm BD-diol (Figure 13) but not in mice and rats exposed to 62.5 ppm BD (Figure 12).

These findings are potentially important because they suggest that circulating concentrations of BD-diol at exposures of 62.5 ppm BD or lower are insufficient to yield significant mutagenic effects in rodents in vivo. This conclusion is supported by the fact that no mutagenic effect was found in female rats exposed for 4 weeks (6 hours/day, 5 days/week) to 62.5 ppm BD (Meng et al.

1999a; Walker and Meng 2000). In contrast, a mutagenic response was found in similarly exposed female mice (Table 5). The species difference is thought to be caused by the formation and circulation of BDO_2 in mice but not in rats at this level of BD exposure (reviewed in Himmelstein and coworkers [1997]).

Comparisons of the mutagenic-potency estimates for female mice exposed to 6, 18, or 36 ppm BD-diol suggest that the mutagenic effects of exposure to this metabolite would plateau at exposure levels exceeding 36 ppm or after exposures to BD yielding equivalent plasma concentrations of BD-diol (Figure 19). The large exposure-related differences in the accumulation of BD-diol in plasma after rodents are exposed to higher than 200 ppm BD (Figure 12) and the comparatively small differences in mutagenic responses after female mice are directly exposed to 6, 18, or 36 ppm BD-diol provide evidence that the contribution of BD-diol–derived metabolites to the overall mutagenicity of BD is confined to relatively high-level BD exposures in rodents. In addition, the accumulation of BD-diol in plasma during active exposures to BD-diol or high-level BD is followed by its rapid elimination in less than 2 hours after exposure to BD. This phenomenon suggests that the small increases in the mutagenic effects of exposure to more than 6 ppm BD-diol (or more than 200 ppm BD) result from (1) exposure-related differences in the plasma concentrations of BD-diol at the end of each 6-hour exposure period and (2) the fact that circulating BD-diol has only a brief window of opportunity to induce mutagenic responses in cells and tissues before it is eliminated.

The inhalation studies of *meso*-BDO_2 and BD-diol in the current investigation, combined with our earlier studies of BD, BDO, and (±)-BDO_2 (Walker and Meng 2000), revealed important factors in species-related mutagenic responses that help clarify the degree to which the various epoxy intermediates contribute to the induction of mutations in rodents at given levels of BD exposure (Table 5). In mice, exposure to 2.5 ppm BDO resulted in the same plasma concentration of BDO as did exposure to 62.5 ppm BD; nevertheless, it had only one-third the mutagenic potency of exposure to 62.5 ppm BD. This indicates that BDO is not a major contributor to the mutagenicity of BD in mice at exposure levels of less than 62.5 ppm BD (Table 5). The mutagenic potency of 62.5 ppm BD in mice could be explained largely by its ultimate metabolism to BDO_2 (regardless of stereochemistry). BDO can also be converted to BDO_2 and BD-diol–derived metabolites; the observed mutagenic responses in mice exposed to 2.5 ppm or 25 ppm BDO (the latter exposure results in plasma concentrations of this metabolite similar to those resulting from exposure to 625 ppm BD) may be caused in large part by its subsequent metabolism to other DNA-reactive metabolites. On the other hand, the mutagenic potency of BDO_2 at exposures that result in plasma concentrations similar to those of exposure to 62.5 ppm BD might account for all of the mutagenic responses in mice exposed to this level of BD (Table 5).

As the exposure level was increased to 625 ppm BD, the identification of the metabolites responsible for the induction of mutations in mice became somewhat more complex. Determining the contribution of BDO_2, for example, at these higher BD levels was complicated by the fact that direct exposures to more than 6 ppm BDO_2 caused respiratory distress in rodents (Henderson et al. 2000). As a result, the mutagenic potency of a BDO_2 exposure that would yield the same plasma concentration as exposure to 625 ppm BD in mice (i.e., approximately 38 ppm BDO_2) could not be estimated. However, the mouse data in Table 5 show that about 40% of the mutagenic response to a 4-week exposure of mice to 625 ppm BD can be attributed to the sum of the mutagenic potencies found for 4-week exposures to 4 ppm (±)-BDO_2 (equivalent to exposure to 62.5 ppm BD) and 36 ppm BD-diol (exposure to 18 ppm BD-diol is equivalent to exposure to 200 ppm BD). Earlier metabolism studies, in which mice were exposed to 71 ppm or 603 ppm BD, showed that the higher exposure produced a threefold increase in blood concentrations of BDO (0.65 and 1.9 µM, respectively). Assuming a linear relation between circulating BDO_2 concentrations and mutagenic responses in mice, then more than 80% of the mutagenic response to exposure to 625 ppm BD can be attributed to the formation of metabolites derived from BDO_2 and BD-diol. (That is: [3 × the 12.7 potency estimate for 4 ppm BDO_2] + [the 12.5 potency estimate for 36 ppm BD-diol] = 50.6; 50.6 ÷ the 61.2 potency estimate for 625 ppm BD = 83% of the mutagenic response.)

In another study, the exposure–response curves of BDO-Gua adducts were linear in mice exposed for 4 weeks (6 hours/day, 5 days/week) to 20–625 ppm BD, but the concentrations of BDO-Gua adducts were magnitudes lower than those for THB-Gua adducts in the same animals (Koc et al. 1999; Swenberg et al. 2001). These data indicate that BDO might contribute in a minor way to the mutagenicity of BD at high-level exposures in mice (as well as rats). Comparisons of data from recently developed assays for BDO_2-specific hemoglobin and DNA adducts with exposure–response patterns for BD-induced M*f*s (Figures 21 and 22) are promising methods for determining more definitively the extent to which each epoxy metabolite contributes to the mutagenicity of BD from low- to high-level exposures in mice and rats.

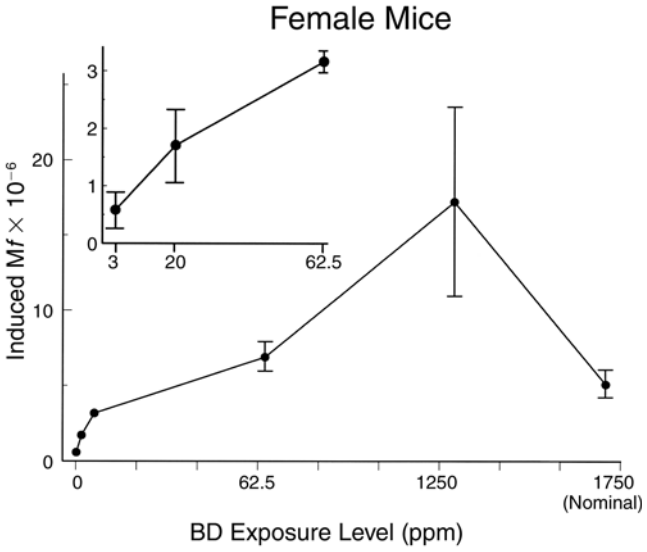

Figure 21. Exposure–response curve for *Hprt* mutant splenic T cells from female mice after inhalation exposure to BD. Animals were necropsied 4 weeks after a 2-week exposure to air or 3 to (nominal) 1750 ppm BD (see section Changes to the Planned Protocol). *Hprt* M*f* data from cells of mice exposed to 20, 62.5, 625, and 1250 ppm BD are taken from earlier studies (Meng et al. 1998a, 1999a; Walker and Meng 2000). The average induced *Hprt* M*f*s (i.e., average observed M*f*s in groups of exposed mice minus the average control values) are plotted against the BD exposure level. Data points are means with error bars (SD). Inset shows enlargement of the lowest 3 data points. To compare points and error bars on the curves: the average *Hprt* M*f* and SD for control animals in the 3- and 1750-ppm BD exposures in this study was $0.96 \pm 0.51 \times 10^{-6}$.

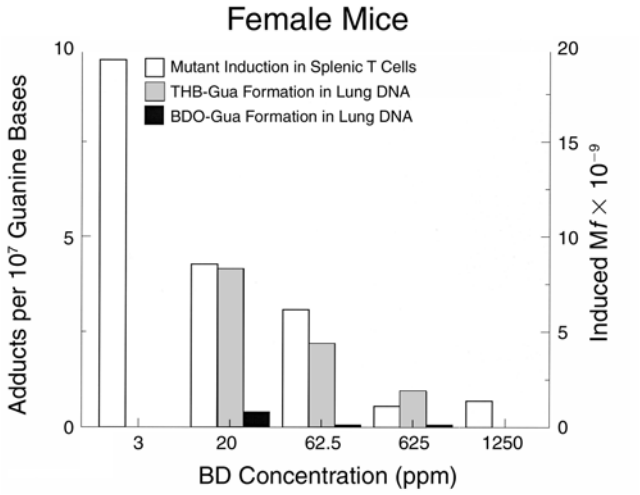

Figure 22. Efficiency of THB-Gua and BDO-Gua adduct formation in lung DNA (as biomarkers of BD exposure) and *Hprt* mutation induction in splenic T-cells (as a biomarker of BD-induced effect) from female mice after inhalation exposure to BD. Mice were exposed to 0 or 3 ppm BD for 2 weeks. Animals were necropsied either 2 hours or 4 weeks after exposure for measurements of DNA adducts (2 hours) or *Hprt* M*f*s (4 weeks). Data for the racemic and *meso* forms of THB-Gua adducts and the BDO-Gua adducts in lung DNA of BD-exposed mice are from Koc and coworkers (1999). *Hprt* M*f* data for mice exposed to 20, 62.5, 625, and 1250 ppm BD are taken from our earlier studies (Meng et al. 1998a, 1999a; Walker and Meng 2000). These comparisons of biomarkers of BD exposure and a biomarker of BD-induced effect do not imply that THB-Gua adducts have a causal role in BD-induced mutagenesis and carcinogenesis. Note that the scales for the *y* and *z* axes differ.

The results of the current investigation also clarify the source(s) of BD-induced mutations in rats. Data in Table 5 for BD- and BD-diol–exposed rats demonstrate that the mutagenic potency of BD-diol–derived metabolites can account for the mutagenic effects in rats exposed to 625 ppm BD. This conclusion is based on the finding that the mutagenic-potency estimate for a 4-week exposure of rats to 36 ppm BD-diol (which results in plasma concentrations of BD-diol that are approximately 25% of those resulting from an exposure to 625 ppm BD) was the same as that for a 4-week exposure to 625 ppm BD (mutagenic-potency estimates of 7.2 for both exposure regimens). Thus, the data summarized in Table 5 add to the body of evidence that the higher rates of BDO_2 formation in mice is the principal cause of the higher rates of BD-induced carcinogenesis in mice versus rats and possibly humans.

EXPOSURE-RELATED PATTERNS IN ADDUCT FORMATION, MUTATION INDUCTION, AND LUNG CANCER INDUCTION IN BD-EXPOSED FEMALE MICE

A plot of the available *Hprt* M*f* data for female mice exposed for 2 weeks to levels of BD ranging from 3 to 1750 ppm (3 ppm and nominal 1750 ppm from the current study; Meng et al. 1999a; Walker and Meng 2000) resulted in a supralinear exposure–response curve (Figure 21); this indicates that BD had more effect at lower exposure levels than at higher levels. This pattern of response may be related to several factors, including lower efficiency of BD bioactivation, higher rates of detoxification, higher levels of DNA repair, and lower efficiency of mutation induction by BD metabolites (related to changes in the ratio of BDO_2 to BDO-diol) as the BD exposure is increased (Meng et al. 1998a, 1999a; Walker and Meng 2000). Conversely, the relatively low mutagenic response in mice exposed to nominal 1750 ppm BD was probably due to substantial cytotoxicity associated with the unplanned exposure regimen of 3500 ppm BD for 3 days followed by 1000 ppm for 7 days (see Methods / Summary of Experimental Protocols and Exposures / Changes to the Planned Protocol, and Meng et al. 2001).

Figure 22 shows BD-induced *Hprt* M*f*s in a manner that illustrates the relative effect per unit dose over the range of BD exposure levels in splenic T cells from female mice. We calculated the efficiency of *Hprt* mutant induction by dividing the induced *Hprt* M*f*s by their respective BD exposure levels and plotting these values against the exposure

levels. These calculations demonstrated that the mutagenic potency of 3 ppm BD was twice that of 20 ppm BD and almost 20 times those for 625 and 1250 ppm BD.

Figure 22 also provides useful comparisons of the efficiency of the formation of the combined racemic and *meso* forms of THB-Gua adducts in lung DNA and the efficiency of the induction of *Hprt* mutant T cells after 2 weeks of exposure of female mice to 3, 20, 62.5, 625, or 1250 ppm BD. As noted earlier, both lung and T cells are target tissues for BD-induced carcinogenesis in mice (Huff et al. 1985; Owen et al. 1987; Melnick et al. 1990; Melnick and Huff 1992). DNA-adduct concentrations have not been measured in T-cell samples from BD-exposed and control mice, but they have been measured (along with frequencies of *Hprt* mutant T cells in the same exposure study) in several tissues, including lung (Koc et al. 1999; Meng et al. 1999a). There was a highly positive correlation between the efficiency of THB-Gua adduct formation and the efficiency of *Hprt* M*f* induction in T cells (as a biomarker of BD-induced effects; Figure 22), suggesting that THB adducts might be good quantitative indicators of mutagenicity in BD-exposed mice. In contrast, there was a very poor correlation between the efficiency of induction of BDO-Gua adducts in lung DNA (and of BDO-induced adducts of *N*-terminal valine of hemoglobin; data not shown) and the efficiency of induction of *Hprt* mutant T cells (Figure 22). The positive correlation does not mean that THB-Gua adducts are responsible for BD-induced mutations. Rather, it implies that the metabolites leading to THB-Gua adducts (i.e., BDO_2 and BDO-diol) are more responsible than BDO for the DNA damage associated with BD-induced mutagenicity and cancer.

Parallel studies of THB-Val and THB-Gua formation in rodents exposed to BD-diol (Powley et al. 2005a,b) and of *Hprt* mutation induction in splenic T cells of BD-diol-exposed rodents in the current investigation demonstrated that the exposure–response curves for THB-adduct formation and *Hprt* mutation induction were remarkably similar in shape. Figure 23 compares the exposure–response curves of the concentrations of THB-Val and the induced *Hprt* M*f*s in female mice exposed to 0, 6, 18, or 36 ppm BD-diol, illustrating that THB-Val adducts track well with mutation induction at the equivalent of high levels of BD exposure (greater than 200 ppm BD) where the BD-diol pathway is mutagenic. (See additional comparisons of exposure–response curves for adducts and induced *Hprt* M*f*s in Powley et al. 2005a.) These data further indicate that THB adducts are good quantitative indicators of BD-induced mutagenicity and suggest that BDO-diol (yielding THB adducts) might be largely responsible for the mutagenic responses in rodents exposed to BD-diol or high levels of BD. That is, BDO-diol causes nearly all the mutagenic effects in rats exposed to high-level BD as well as the mutagenic effects beyond those produced by BDO_2 in mice exposed to high-level BD. These observations highlight the great need to develop assays sensitive enough to distinguish between the DNA adducts formed by BDO_2 and BDO-diol, and for additional work to determine more definitively the relative contribution of these and other BD metabolites to the DNA alkylation and mutation patterns observed in mice and other species (Walker and Meng 2000).

On the other hand, earlier studies of metabolite-specific *Hprt* mutations in mice demonstrated that the mutagenicity of BD at low exposure levels (20 or 62.5 ppm) could be attributed to BDO_2 (Meng et al. 1999a,b). It is conceivable that the mutagenic effects observed in mice exposed to 3 ppm BD can be accounted for almost exclusively by the in vivo production of BDO_2. Thus, it is important to measure concentrations of BD metabolites and adducts in the blood and tissues of mice exposed to low levels of BD in the range of workplace exposures. Also, molecular analyses of *Hprt* mutant T cell clones from mice exposed to 3 ppm BD (versus controls) could provide further evidence of BD mutagenesis at low exposure levels and the potential role of BDO_2.

A comparison of the efficiency of *Hprt* mutant T-cell induction after short-term low-level BD exposures (2 weeks; $\leq$ 62.5 ppm) to the efficiency of lung-tumor induction after long-term BD exposures (2 years; $\leq$ 62.5 ppm) suggests that exposures of female mice to 3 ppm BD under cancer-bioassay conditions (similar to those used by the National Toxicology Program) would yield significant increases in

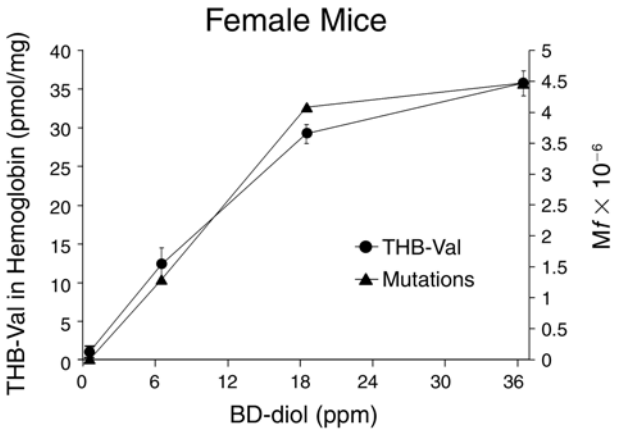

Figure 23. Exposure–response curves for formation of THB-Val and for induced *Hprt* mutant splenic T cells of female mice exposed to BD-diol. Mice were exposed by inhalation to 0, 6, 18, or 36 ppm BD-diol for 4 weeks (6 hours/day, 5 days/week; Specific Aim 4, Inhalation Studies C and F). Animals were necropsied either 2 hours or 4 weeks after exposure for measurements of THB-Val (2 hours) or *Hprt* M*f*s (4 weeks). Data points are means with SD error bars. Note that the scales for the *y* and *z* axes differ.

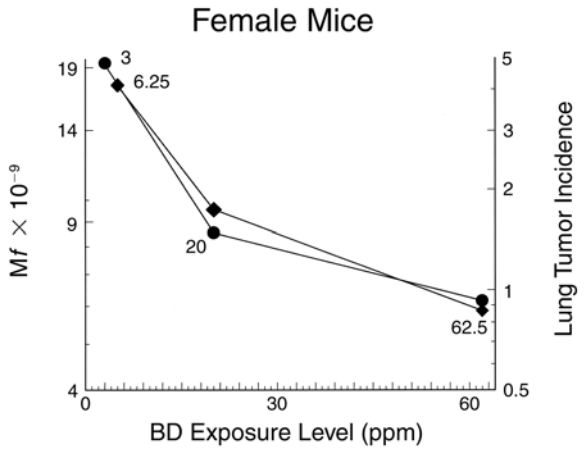

Figure 24. Efficiency of induction of *Hprt* mutant splenic T cells and induction of lung tumors in female mice after inhalation exposure to BD. For measurement of *Hprt* M*f*s (*y* axis), control and exposed animals were necropsied 4 weeks after a 2-week exposure to 0, 3, 20, or 62.5 ppm BD (current study [0 and 3 ppm]; Meng et al. 1998a, 1999a; Walker and Meng 2000). For carcinogenesis studies (*z* axis), animals were exposed 0, 6.25, 20, or 62.5 ppm BD for up to 2 years (Melnick et al. 1990; Melnick and Huff 1992). The incidence of BD-induced lung tumors at a given exposure level is: [number of BD-exposed mice with a lung tumor] − [number of control mice with a lung tumor]. ● is mutagenic efficiency at *Hprt* of splenic T cells; ◆ is carcinogenic efficiency in mouse lung (these data are from Table 6 in Melnick and Huff 1992). (Note that control animal data were not plotted in this graph because the M*f*s and lung cancer incidence values for control animals were subtracted from the corresponding data for BD-exposed animals, then divided by the exposure level, to derive mutagenic and carcinogenic efficiency estimates, respectively, for each BD exposure level.) Data on *y* and *z* axes are log scale.

lung tumors (Figure 24). This possibility is supported by an earlier report by Melnick and Huff (1992), in which extrapolation of the exposure–response curve for lung cancers from BD exposure as low as 2 ppm resulted in an estimated twofold increase in lung cancers in female mice. Nonetheless, the magnitude of the BD-induced mutagenic response in female mice after 2 weeks of exposure to 3 ppm BD was small (but significant) compared with background M*f* values. *Hprt* M*f*s were previously shown to increase over time during 4 weeks of exposure of female mice and rats to high levels of BD (Meng et al. 1998a, 1999a). But it is not known for how long (beyond 4 weeks of exposure) mutations would accumulate before reaching a plateau (Walker and Meng 2000). Data from these low- and high-level BD mutagenicity studies (work reported here; Meng et al. 1998a, 1999a; Walker and Meng 2000), combined with cancer-bioassay results, suggest that, although the initial increases in M*f*s after short-term exposures provide useful exposure–response information, it is the "mutation load," or elevation in M*f* over background for an extended period, that is the critical determinant in the cancer risk in mice exposed long-term to low levels of BD.

COMPARISON OF MUTATION-SPECTRA DATA FOR BD AND ITS EPOXY METABOLITES

Earlier efforts to explore the mechanisms of BD-induced mutagenicity and carcinogenicity involved mutation spectra studies of BD, BDO, BDO_2, and BDO-diol using a number of mutation targets: the *lacI* gene of Rat2 cells (Recio et al. 2001) and of exposed transgenic mice (Recio et al. 1996; Saranko et al. 2001); as well as the *Hprt* gene of CHO-K1 cells (Lee et al. 2002), of human lymphoblastoid cells (Cochrane and Skopek 1994a; Steen et al. 1997a,b; Recio et al. 2001), and of exposed mice, rats, and humans (Cochrane and Skopek 1994b; Meng et al. 1999a, 2000; Ma et al. 2000; Table 13). Each of these targets has characteristic limitations; but the predominance of base substitutions at A•T sites after in vitro or in vivo exposures to BD, BDO, or BDO_2, and the occurrence of deletion mutations after in vitro or in vivo exposure to BD or BDO_2 have been much emphasized. Several investigations also sought to identify exposure-related point mutations in oncogenes and tumor-suppressor genes of neoplasms from BD-exposed rodents (Goodrow et al. 1990; Sills et al. 1999). The common features of these studies were that (1) only base substitutions and micro-deletions or micro-insertions were characterized, and (2) large deletions and chromosomal rearrangements could not be defined because of limitations in the experimental systems and analytical methods used. In the current investigation, a multiplex PCR method was developed and used in parallel with RT–PCR and cDNA sequencing for the first coincident identification of both large- and small-scale genetic alterations in *Hprt* of BD-exposed and control mice. Established multiplex PCR and RT–PCR methods (Chen et al. 1999) were also used to define more comprehensive *Hprt* mutation spectra for BD-exposed and control rats.

For the current mutation-spectra experiments, mutant T-cell clones were collected from male mice and rats and expanded so that molecular analyses could be performed using both *Hprt* cDNA and genomic DNA to identify small- and large-scale mutations. Quantitative PCR methods have yet to be developed for the *Hprt* of any species, including humans. However, the second, inactive copy of *Hprt* on the X chromosome in female mice and rats precludes the use of multiplex PCR to analyze mutant genomic DNA and thereby limits the range of mutation-spectra data that can be obtained. A total of 279 *Hprt* mutants from either control or BD-exposed male mice were first analyzed by RT–PCR. Some expanded mutants from control mice (24 of 111 expanded clones) and BD-exposed mice (18 of 168 expanded clones) did not yield a visible cDNA product and were later shown not to have genomic DNA deletions. (Similar observations were obtained using rat *Hprt* mutant

Table 13. Significant Findings in Mutation-Spectra Data from Studies with Different Exposure Protocols and Target Mutations for Exposure to BD and Its Epoxy Metabolites[a]

	BD in Vivo Exposures[b]						BDO in Vitro or in Vivo Exposures[b,c]			BDO_2 in Vitro or in Vivo Exposures[b,d]			BDO-diol in Vitro Exposure[b]
Type of Mutation	Human *HPRT*	Human *HPRT*	Rat *Hprt*	Mouse *Hprt*	Mouse Exon 3	Mouse *lacI*	Human *HPRT*	Mouse *lacI*	Rat2 *lacI*	CHO-K1 *Hprt*	Human *HPRT*	Mouse Exon 3	CHO-K1 *Hprt*
Footnote	E	F	G	G	H	I	J	I	J	K	J	H	K
Base Substitutions													
G•C → A•T				*	*	*	*	*	*	*		*	*
A•T → G•C			*		*	*						*	*
G•C → T•A				*	*	*			*				
G•C → C•G				*	*							*	
A•T → C•G			*	*	*				*			*	
A•T → T•A			*	*	*	*	*		*	*	*	*	
Frameshifts				*									
+ 1			*	*									
− 1		*		*									
Deletions		*	*	*	*				*	*	*		*
5′ Partial			*	*									
3′ Partial				*									
Internal				*									
Complete			*	*									
Insertions													
Splicing errors			*										
Complex			*		*								

[a] An * indicates a significant increase over the control value.

[b] Due to the lack of concurrent control data, findings from some studies were not included: [1] mutation-spectra findings at the *Hprt* or *Tk* loci of mice exposed to BD, BDO, or BDO_2, or in TK6 cells exposed in vitro to BDO, BDO_2, or BDO-diol (from Cochrane and Skopek 1994a,b); and [2] mutation-spectra data at *Hprt* exon 3 of rats exposed to BDO_2 (from Meng et al. 2000).

[c] Molecular analyses of rodent *Hprt* mutants were not performed because BDO exposures of mice were weakly mutagenic compared with control mice, and too few mutants were recovered from BDO-exposed mice to make mutation-spectra studies possible (Meng et al. 1999b).

[d] Molecular analyses of *lacI* mutants were not performed in transgenic mice or in Rat2 cells (reviewed in Recio et al. 2001) after BDO_2 exposures because induced M*f*s were not greater than control values. However, BDO_2 exposure was associated with exposure–response increases in micronuclei in Rat2 cells.

[e] In findings from Albertini and associates (2003), the molecular spectra of the *HPRT* mutant isolates recovered from BD-exposed workers were not statistically different from the spectra of mutations identified in the unexposed control group. Furthermore, all spectra in groups of BD-exposed workers were statistically similar to what other researchers have observed historically for adults who have not been exposed to mutagens.

[f] Findings from BD-exposed workers (Ma et al. 2000) showed a significant increase in the frequency of deletion mutations, particularly multiple exon deletions, compared with control subjects. Locations of the deletions within the *HPRT* gene were not reported.

[g] Findings shown in Tables 11 and 12 from the current study.

[h] Findings from reanalysis (conducted for this study) of spectra data for the mutation reported in the exon 3 region of the *Hprt* gene in T cells from mice exposed to BD or BDO_2 compared with controls (Meng et al. 2000).

[i] Findings in the *lacI* gene of transgenic mice exposed to BD or BDO (reviewed in Recio et al. 2001). The increase over background in G•C → A•T transitions were significant for those mutations that occurred at non-CpG dinucleotides in BD-exposed mice and at CpG sites in BDO-exposed mice.

[j] Findings in the *HPRT* gene of TK6 human lymphoblastoid cells, or the *lacI* gene of Rat2 cells, after cells were exposed in vitro to BDO or BDO_2 (reviewed in Recio et al. 2001). The increase over background in G•C → A•T transitions was significant for those mutations that occurred at non-CpG sites in BDO-exposed Rat2 cells.

[k] Findings are based on statistical analysis of raw spectra data for *Hprt* mutations in CHO-K1 cells exposed in vitro to BDO_2, BDO-diol, or vehicle alone (Lee et al. 2002).

colonies.) The lack of cDNA products from this limited number of clones might have been caused by point mutations that affect RT–PCR primer binding regions, poor-quality RNA, or simply a reaction failure. The most likely reason for failing to obtain cDNA products from these colonies was that they were expanded in culture to the point where the cells stopped growing and expression of the *Hprt* and K-*ras* genes had diminished. The resulting reduction in mRNA would preclude reverse transcription and identification of base substitutions or micro-deletions and micro-insertions (if present) in genomic DNA. During the expansion of *Hprt* mutant clones from mice and rats, timely monitoring and harvesting of clones during active cell growth are critical for obtaining good-quality total RNA for molecular analysis.

A fairly high number of mouse and rat cDNAs from presumptive *Hprt* mutants had wild-type sequences in amplified cDNA products, a phenomenon that was previously observed in 6-TG–resistant clones from control and thiopeta-exposed rats (Chen et al. 1999). Several explanations for this finding are possible, but the occurrence of expanded clones with wild-type sequences in the current investigation is most likely the consequence of changes in the protocol used for harvesting and expanding 6-TG–resistant colonies. Microtiter dishes were scored for colony growth in the presence of 6-TG at 8 to 9 days after plating (Meng et al. 2001) instead of 10 to 12 days after plating (Walker and Skopek 1993; Meng et al. 1998a,b) in order to collect positive colonies as early as possible for mitogenic stimulation and outgrowth. Expansion of these positive colonies was carried out in supplemented medium without 6-TG; a few wild-type cells that may have survived the reduced 6-TG selection period might have had a growth advantage during colony expansion, which could have masked the mutation occurring in the cells scored as 6-TG–resistant. Other possible explanations include the occurrence of (1) defects in another step in the nucleotide-salvage pathway leading to 6-TG–resistance, (2) mismatch-repair-deficient cells expressing 6-TG–resistance (Fink et al. 1998), (3) silencing the *Hprt* gene via promoter-region mutations or hypermethylation (Chen et al. 2001), (4) factors that might reduce message stability but not the coding sequence in DNA, and (5) misidentification of positive clones during scoring. Additional research is needed to clarify the methodological shortcomings or mechanisms that led to finding 6-TG–resistant colonies with wild-type *Hprt* sequences after clonal expansion.

Our research team has used several approaches to characterize the mutations induced by BD (or BDO_2) exposure in B6C3F1 mice and F344 rats, including analysis of the *Hprt* exon 3 region using (1) PCR amplification of genomic DNA and denaturing gradient gel electrophoresis followed by sequence analysis of mutants from female mice and rats (Meng et al. 2000); (2) RT–PCR with sequencing of cDNA of mutants from female mice (Meng et al. 1999a); and (3) in this study a combination of RT–PCR and cDNA sequencing plus multiplex PCR of genomic DNA to identify mutations in control and BD-exposed male mice and rats. The main findings in BD-exposed female mice were (1) significant increases in the mutant fractions of all types of base substitutions in *Hprt* exon 3 (including a dramatic increase in the percentage of G•C → C•G transversions from 5% of total mutants in control mice to 32% in exposed mice; Meng et al. 2000) and (2) complete *Hprt* deletions in mutants from control and exposed mice (12% and 30%, respectively, of total mutants; Meng et al. 1999a).

In male mice, the nature of the significant findings in BD-exposed animals compared with controls varied to some extent depending on whether the mutation-spectra data were obtained by RT–PCR analysis and sequencing of cDNA, by multiplex PCR of genomic DNA, or by combining the two approaches. RT–PCR analysis and cDNA sequencing revealed a statistically significant difference between the overall proportions of the types of mutations detected in control and exposed mice ($P = 0.042$; Table 10). Multiplex PCR of genomic DNA showed that deletion mutations as a whole were significantly increased over background in exposed mice ($P = 0.031$). After combining the two data sets (Table 11), the statistical difference between the proportions of large- and small-scale mutations in exposed and control mice just missed being significant ($P = 0.061$). However, statistical analysis of the mutant fractions for individual mutation types occurring in exposed and control mice demonstrated that BD exposure significantly increased the frequency of each type of base substitution except for A•T → G•C transitions (G•C → C•G transversions were the most frequently induced) and caused considerable increases in frameshifts (13% of the mutagenic response) and deletions (52% of the mutagenic response) in male mice (Table 11).

Analysis of cDNA-sequence data alone or combined with multiplex PCR data showed that there was no difference between the overall mutation spectra for BD-exposed and control male rats. Yet statistical comparisons of the mutant fractions for individual types of *Hprt* mutations in control and exposed rats demonstrated that BD exposure was associated with significant increases in several types of base substitutions at A•T bp, single-base insertions, deletion mutations, and complex mutations (Table 12).

Significant increases in different types of base substitutions were not uniformly found in male rodents exposed to BD. However increases in (1) the mutant fractions of all

types of base substitutions except A•T → G•C transitions in exposed male mice (Table 11) and (2) the mutant fractions of base substitutions at A•T bp in exposed male rats (Table 12) were consistent with significant findings in one or more of the mutational targets that have been examined in earlier studies of BD and its mutagenic metabolites (Table 13). Significant increases in the frequencies of G•C → A•T transitions, A•T → G•C transitions, and A•T → T•A transversions have been found in one or more tissues of B6C3F1 *lacI* transgenic mice exposed to BD (Recio et al. 2001).

BDO exposure increased the frequency of A•T → T•A transversions at the *lacI* loci in Rat2 cells and at the *HPRT* loci in human TK6 lymphoblastoid cells; whereas BDO_2 exposure of human TK6 lymphoblastoid cells increased the frequency of A•T → T•A transversions at *HPRT* (Recio et al. 2001). BDO_2 exposure of CHO-K1 cells increased the frequency of G•C → A•T transitions and A•T → T•A transversions at the *Hprt* locus; whereas BDO-diol exposure increased the frequency of G•C → A•T and A•T → G•C transitions in the same gene compared with control cells (Lee et al. 2002). In vivo BDO_2 exposures produced increased frequencies in the mutant fractions of all types of base substitutions in the exon 3 region of *Hprt* in T cells of exposed female mice (Meng et al. 2004). As noted above, significant increases in G•C → C•G transversions were also found in BD-exposed female mice (Meng et al. 2000) at a higher proportion than those reported here in similarly exposed male mice. Yet, G•C → A•T transitions and A•T → T•A transversions were the base substitutions found most consistently across the different experimental approaches used in mutation-spectra studies of BD, BDO, BDO_2, and BDO-diol (Table 13).

To date, G•C → C•G and A•T → T•A transversions are the only base substitutions that have been demonstrated to have unquestionable biologic relevance to BD-induced cancer. G•C → C•G transversions have previously been found as activating mutations in (1) codon 13 of the K-*ras* gene of neoplasms of the forestomach, Harderian gland, liver, lung, and lymphocytes in BD-exposed mice (Goodrow et al. 1990, 1994; Hong et al. 1997; Zhuang et al. 1997; Sills et al. 1999, 2001), and (2) in codon 13 of the H-*ras* gene of mammary-gland tumors in BD-exposed mice (Zhuang et al. 2002). Also in BD-exposed mice, A•T → T•A transversions have been observed as activating lesions in codon 61 of the H-*ras* gene or the K-*ras* gene (or both) in forestomach, Harderian gland, liver, and lymphocytes (Table 14). A•T → G•C transitions were also found as activating mutations in H-*ras* codon 61 of forestomach and Harderian-gland tumors from BD-exposed mice but not at higher frequencies than in neoplasms from control animals (Sills et al. 1999, 2001). These findings do not take into consideration the fact that (1) G•C → C•G and A•T → T•A transversions are only two types of changes that can

Table 14. Principal *ras* Mutations Detected in Tumors from BD-Exposed Mice[a]

	H-*ras* codon 13 (Normal = GGC)	H-*ras* codon 61 (Normal = CAA)		K-*ras* codon 13 (Normal = GGC)	K-*ras* codon 61 (Normal = CAA)
Tissue	G → C	A → T	A → G	G → C	A → T
Forestomach[b]	ND	+[c]	+	+[c]	ND
Harderian gland[d]	ND	+	+	+	+
Liver[e]	ND	+	—	+	+
Lung[e]	ND	—	—	+[c]	—
Lymphocytes[f]	ND	—	—	+	+
Mammary gland[g]	+	—	—	—	—

[a] A + indicates mutations were found, and a — indicates they were not; ND indicates not done (assay not performed).

[b] Data are from Sills et al. 2001.

[c] Increased frequency of occurrence compared with control mouse neoplasms, though the difference was not significant due to small sample sizes. For all other mutations in this table, either control neoplasms were not evaluated or the proportions of neoplasms with specific mutations occurred in similar frequencies in exposed and control mice.

[d] Data are from Goodrow et al. 1994, Hong et al. 1997, and Sills et al. 1999.

[e] Data are from Goodrow et al. 1990.

[f] Data are from Goodrow et al. 1990 and Zhuang et al. 1997.

[g] Data are from Zhuang et al. 2002.

activate K-*ras* genes during the selective pressure associated with the chemical induction of mouse lymphomas and other cancers (Sills et al. 2001); (2) G•C → C•G and A•T → T•A transversions are unlikely to be the only critical mutations in BD-induced tumorigenesis; and (3) G•C → C•G and A•T → T•A transversions might be important activating lesions caused by other epoxide or epoxide-forming chemicals that induce similar types of tumors in mice (e.g., chloroprene and isoprene; Sills et al. 2001).

It is remarkable that A•T → T•A transversions occurred at similar mutant fractions in the *Hprt* gene of BD-exposed mice and rats, whereas G•C → C•G transversions were significantly increased only in mice exposed to BDO_2 (Meng et al. 2000) or to BD (current study; Tables 11, 12, and 13). BD-exposed mice produce substantial amounts of BDO_2,whereas BD-exposed rats produce little to none (Himmelstein et al. 1994, 1995, 1996; Thornton-Manning et al. 1995a,b, 1996). These data suggest that the biotransformation of BD to BDO_2 might be one source of BD-induced G•C → C•G transversions. In addition, HMVK forms 1,N^2-propanodeoxyguanosine adducts in vitro that might also cause G•C → C•G transversions. Additional research is needed to determine whether the in vivo formation and persistence of HMVK adducts after high-level BD (or direct BD-diol) exposure is sufficiently different in mice and rats to cause G•C → C•G transversions in mice but not in rats. Discovering the metabolites that ultimately induce G•C → C•G and/or A•T → T•A transversions in reporter genes and protooncogenes remains an important goal that will help clarify species differences in the cancer risk of BD exposure.

An increase in frameshift mutations, like that found in BD-exposed male mice, was previously observed in exon 3 of *Hprt* in splenic T cells of mice exposed to BD or its epoxy metabolites (Cochrane and Skopek 1994b) or to ethylene oxide (Walker and Skopek 1993). Ma and associates (2000) reported a significant increase of −1 frameshifts in the *HPRT* gene of peripheral lymphocytes in BD-exposed workers (11% vs. 2% in controls). Walker and Skopek (1993) proposed that DNA adducts formed at ring nitrogens by ethylene oxide or BD-reactive epoxides produce apurinic sites that, when located in runs of consecutive guanine or adenine, might cause the DNA polymerase to "hesitate," allowing strand slippage to occur and lead to frameshift mutations. This hypothesis is supported in the current investigation by the fact that most of the frameshift mutations occurred at runs of consecutive guanines or adenines.

Comparisons of various mutation-spectra studies of BD exposure (Table 13) demonstrate that our approach—that is, analyzing both large- and small-scale genetic alterations—more clearly reveals the broad spectrum of mutations induced and the magnitude of the various types of deletions in both mice and rats. At first glance, the fact that deletions taken together constituted more than 50% of all induced mutations in BD-exposed rats (Table 12) appears surprising, because the clastogenic properties of BD reported for mice but not observed in rats had been attributed largely to its conversion to BDO_2, a bifunctional alkylating agent capable of forming DNA–DNA and DNA–protein cross-links (Recio et al. 2001). In related studies, Cochrane and Skopek (1994a) showed by Southern blot analysis that 54% of *HPRT* mutants from BDO_2-exposed human TK6 cells had partial or complete gene deletions; and Steen and coworkers (1997a) used genomic amplification to demonstrate a significant increase in 5′ partial losses of the *HPRT* gene in BDO_2-exposed human TK6 cells. In BD-exposed workers, the frequency of multiple *HPRT* exon deletions increased 2.1-fold compared with that in control subjects (Ma et al. 2000), although the locations of the deletions in the *HPRT* gene were not reported. Yet it might be that humans produce little to no BDO_2 after BD exposure (International Agency for Research on Cancer 1992). This possibility appears to be supported by recent *HPRT* mutation-spectra studies of Czech workers, in which no evidence was found of any exposure–related BD-induced genetic effects in exposed groups (Albertini et al. 2003).

Studies comparing the mutagenic potency (represented by the difference between the areas under the *Hprt* mutant T-cell manifestation curves for exposed and control rodents) of BDO, BDO_2, and BD-diol to that of BD, when comparable blood concentrations of metabolites were reached, provide strong evidence that (1) the formation of BDO-diol accounts for most of the mutagenicity of BD in rats and (2) BDO_2 and BDO-diol account (to differing degrees) for the mutagenicity of BD in mice as the BD exposure level is varied (current study). It is reasonable to hypothesize that deletion mutations in the *Hprt* gene of BD-exposed rats might arise from BDO-diol in a manner similar to the DNA-strand breaks and deletions induced by the simplest of the epoxides, ethylene oxide. In BD-exposed mice, BDO-diol and BDO_2 might both cause deletions, though probably only BDO_2 is responsible for the additional large-scale mutation events associated with the clastogenicity of BD in mice. The likelihood that BDO-diol exposure produces deletion mutations is supported by the recent finding that exposure of CHO-K1 cells to BDO-diol or BDO_2 results in similar significant increases in exon deletions among the *Hprt* mutations found (i.e., 39% and 38%, respectively, compared with 18% in control cells; Lee et al. 2002).

IMPLICATIONS OF THE FINDINGS

The overriding goal of our BD research has been to understand the mechanisms by which BD, through its DNA-reactive metabolites, induces the formation of adducts, mutations, and tumors in mice and rats so that this information can be used to inform investigations of human responses to BD and be incorporated into a mechanism-based risk-assessment model for exposed humans. A better understanding of the role of specific BD metabolites in the induction of DNA damage in rodents will make it possible to assess the risk stemming from the formation of these metabolites in humans. Because of the important role of BDO_2 in the induction of DNA damage in mice, especially in female mice, additional information needs to be obtained about the formation of BDO_2 in mice, rats, and humans by evaluating exposed animals and individuals for the presence of biomarkers specific for this metabolite in blood and urine. The results of this combined body of work will make it possible to interpret the health risks for humans of low-level BD exposures in the range of current occupational limits.

The evidence reported here — that repeated (4-week) low-level exposure to 3 ppm BD is mutagenic in female mice — should not be surprising because it is known that lung cancer occurs in female mice exposed to 6 ppm BD. On the other hand, the M*f* data for mice do not mean that low-level BD exposure would produce the same mutagenic effects in people. Instead, the exposure–response relations for biomarkers of exposure and effect in rodents ultimately need to be compared with biomarker data obtained in population studies of BD workers (Hayes et al. 1996; Tates et al. 1996; Ward et al. 1996a,b; Ma et al. 2000; Albertini et al. 2003) to improve cancer risk assessment in humans. Thus, better delineation of the changes in biomarkers in rodents after low-level BD exposures (at and below occupational exposure levels) will be an important area of future investigation.

Current and past work has demonstrated that much of the mutagenicity of BD at low levels might be attributable to the metabolite BDO_2; in mice, essentially all the mutagenicity of BD at low levels might be attributable to this metabolite. Other metabolites appear to affect the *Hprt* M*f* in mice as BD-exposure levels increase. In rats, data reported here indicate that BD-diol–derived metabolites might account for the mutagenicity and carcinogenicity that occur only at high levels of BD exposure (i.e., > 200 ppm BD). These discoveries support the need for new studies designed to measure concentrations of BD metabolites, adducts in blood and tissues, and reporter-gene mutations in mice exposed to BD at workplace levels of exposure and lower.

Work of this kind has become critically important because the latest risk assessment by the U.S. Environmental Protection Agency (U.S. EPA) estimates that exposure to 0.01 ppb BD would produce one cancer per million individuals (U.S. EPA 2002); this assessment was based largely on cancer data from BD-exposed female mice and the use of the default linear, no-threshold model. On the other hand, because various methods can screen for and keep the number of spontaneous mutations in check (Loeb 1989), an important question needs to be addressed in future BD-mutagenicity studies: Does the supralinear exposure–response curve for exposure to 3 to 62.5 ppm BD (Figure 6) adequately predict, or overestimate, induced *Hprt* M*f*s for much lower exposures, such as 0.1 to 1.0 ppm BD? It is possible that a threshold for the induction of BD-induced mutations in mice might be identified, which would limit the application of the default linear, no-threshold model. It is also possible that, based on future species-specific biomarker data, the rat will prove to be a better model than the mouse for assessing the human health risks of BD exposure.

The findings of our investigations demonstrate the utility of mutagenic-potency data alone or in combination with adduct data in drawing conclusions about the causative intermediates and underlying mechanisms of BD-induced mutagenesis and carcinogenesis. Surprisingly, our studies of the stereospecific forms of BDO_2 showed that its stereochemistry did not significantly affect its overall mutagenicity. It now seems unlikely that the stereochemistry of BDO and BDO-diol will affect their mutagenicity as well. The results of our collaborative studies (Koc et al. 1999; Health Effects Institute 2000; Powley et al. 2005a,b, 2007) further showed strong correlations between exposure–related increases in THB-Val formation in hemoglobin, THB-Gua adduct formation in lung, *Hprt*-mutation induction in lymphocytes, and the incidence of lung cancer induction in BD-exposed female mice. Likewise, the exposure–response curves for THB adduct formation and *Hprt* mutation induction were similar in shape after direct exposure of female mice and rats to BD-diol, suggesting a prominent role for BDO-diol in mutagenesis at high-level BD exposures (Powley et al. 2005a). These positive correlations illustrate the wealth of mechanistic information that can be gained by comparing patterns of adduct formation and mutation induction in rodents exposed to BD and its individual metabolites. These correlations also support the call for studies of the potential roles of BDO_2 and BDO-diol in the induction of DNA damage associated with lung cancer in mice. It will be necessary, for example, to identify the adduct(s) specific to BDO_2, BDO-diol, and HMVK so that the relative roles of these and other metabolites in

BD-induced DNA alkylation, and the mechanisms underlying the mutagenic responses in rodents, can be determined more definitively at low- and high-level exposures.

The *Hprt* mutation-spectra studies completed to date have provided some unexpected information about the specificity of the classes of BD-induced mutations found in rodents. For example, the induction of deletion mutations and base substitutions only at A•T bp after rats were exposed to BD was somewhat unexpected. Extending the analysis of large- and small-scale mutations to mice and rats exposed to BDO_2 or BD-diol (leading to mutations induced by BDO-diol and perhaps by HMVK) should help identify species-related differences in the sources of deletion mutations and in the overall types of base substitutions induced by BD exposure. Therefore, it is important to understand the potential value of mutation-spectra data from mice and rats exposed to BD-diol and BDO_2 in identifying the relative (and species-specific) contributions of these metabolites to mutagenesis at high-level BD exposures.

Previous molecular studies using the *Hprt* gene or *lacI* transgene have clearly shown that BD exposure increases the frequency of various mutation types that occur spontaneously in control animals. However, there is no classical mutation fingerprint that would highlight one or more hotspots for mutations, or in which increases in one or two types of mutations would stand out; if there were, mutagenesis could be connected unequivocally with BD exposure. On the other hand, high-level BD exposure (e.g., 1250 ppm BD for 2 weeks) does yield a fingerprint in terms of the mutant fractions (fractions of particular types of mutations) that contribute in a statistically significant manner to the total chemically induced increase in M*f* (compared with control values). High-level BD exposure in both mice and rats significantly increases the frequency of certain types of mutations compared with the frequency found in control animals. The significantly induced mutant fractions for certain types of mutations are sufficiently different in BD-exposed mice and rats to permit fruitful comparisons between mutation-spectra data for BD and its DNA-reactive metabolites.

Comparisons of the mutation-spectra data for rodents exposed to BDO_2 or BD-diol and to BD will undoubtedly add to the weight of evidence implicating these metabolites in causing certain species-related mutations in mice and rats exposed only to BD. Both BDO_2 and the BD-diol pathway are expected to induce significant numbers of deletions and frameshifts (Table 13). Therefore, it might be the differences in the types of base substitutions (and the concentrations of metabolites and adducts formed in vivo) that distinguish the mutagenic specificity of reactive intermediates derived from BDO_2 and BD-diol in mice and rats exposed directly to the two metabolites (instead of to BD). G•C → C•G and A•T → T•A transversions are critical mutations because they activate proto-oncogenes in cancers in BD-exposed mice; therefore, mutation-spectra studies could help clarify species-related differences in cancer risk by exploring why significant increases in BD-induced base substitutions occur at both G•C and A•T bp in mice but only at A•T bp in rats. Based on species-related differences in metabolism and the results of earlier mutagenic-potency and mutation-spectra studies of BD, it is reasonable to predict that BDO_2 causes base substitutions at both G•C and A•T bp in BD-exposed mice, whereas BD-exposed rats do not produce enough BDO_2 to cause a significant increase in base substitutions at G•C bp. On the other hand, adducts derived from the metabolism of BD-diol to BDO-diol are likely to cause base substitutions at A•T bp but not G•C bp at high BD exposure levels (≥ 200 ppm) in both rodent species. The observed mutagenic specificity of BD and the predicted mutagenic specificity of BDO_2 and BD-diol in mice and rats are summarized in Table 15. A key question is whether G•C → C•G transversions arise solely from BDO_2-induced adducts or possibly from HMVK-induced adducts as well. Studies to address this question would allow unprecedented comparisons of small- and large-scale mutations occurring across the *Hprt* genes of mice and rats exposed to BD metabolites with those of mice, rats, and humans exposed to BD.

Ongoing and future studies of patterns in DNA alkylation and mutation in mice and rats should provide a better basis for predicting the relative contributions of the various BD metabolites to BD-induced mutagenesis and carcinogenesis in experimental models. Considering the important role of gross deletions or loss of heterozygosity in carcinogenesis, developing biomarkers specifically for BDO_2 exposure deserves special attention; such biomarkers would enable

Table 15. Observed or Predicted Base Substitutions in Exposed Mice and Rats

	Expected Locations of Base Substitutions	
Compound	Mice	Rats
BD[a]	G•C and A•T	A•T
BDO_2[b]	G•C and A•T	G•C and A•T
BD-diol[b]	A•T	A•T

[a] Based on mutation-spectra data from BD-exposed male mice and rats (Specific Aim 5, current study; Meng et al. 2000).

[b] Base substitutions are predicated on the supposition that metabolism of BD-diol to HMVK does not differ sufficiently between species to yield mutations at G•C bp in mice but not in rats.

us to monitor and evaluate cancer risk in humans exposed to BD. These directions should be advanced by (1) the recent development of methods to detect BDO_2-specific hemoglobin adducts (Georgieva et al. 2004) and a BDO_2-specific DNA cross-link adduct (N7-guanine–N7-guanine; Park and Tretyakova 2004); and (2) the presentation here of (a) substantive mutagenic-potency data for rodents exposed to BD and BD metabolites, and (b) mutation-spectra data for both large- and small-scale mutations in exposed rodents. This information will facilitate comparing adduct and molecular data in future studies of BD and its metabolites.

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ABBREVIATIONS AND OTHER TERMS

AMV	avian myeloblastosis virus
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
BDO-Gua	hydroxybutenyl adducts at N7 of guanine
bp	base pair or pairs
cDNA	complementary DNA
CE	cloning efficiency
CHO	Chinese hamster ovary
CHO-K1	line of Chinese hamster ovary cells
DMDTC	dimethyldithiocarbamate
DOE	U.S. Department of Energy
EH	epoxide hydrolase
U.S. EPA	U.S. Environmental Protection Agency
GC	gas chromatography
GC/MS	gas chromatography and mass spectrometry
GST	glutathione *S*-transferase
HCl	hydrochloric acid
HMVK	hydroxymethylvinyl ketone
Hprt	rodent hypoxanthine-guanine phosphoribosyl transferase gene
HPRT	human hypoxanthine-guanine phosphoribosyl transferase gene
H-*ras*	a proto-oncogene
K-*ras*	a proto-oncogene
lacI	the fourth *lac* gene, encoding the lactose repressor
meso-BDO_2	an achiral stereoisomer of BDO_2
M*f*	mutant frequency
M1	*N*-acetyl-*S*-(3,4-dihydroxybutyl)-L-cysteine
M2	isomeric mixture of the regio- and stereoisomers (*R*)/(*S*)-*N*-acetyl-*S*-(1-(hydroxymethyl)-2-propenyl)-L-cysteine and (*R*)/(*S*)-*N*-acetyl-*S*-(2-hydroxy-3-butenyl)-L-cysteine
NMR	nuclear magnetic resonance spectroscopy
P450	cytochrome P450
PCR	polymerase chain reaction
ras	gene that encodes for the ras protein
Rat2 cell	Rat2 *lacI* transgenic fibroblast
RT–PCR	reverse transcription–polymerase chain reaction
SBR	styrene-butadiene rubber
6-TG	6-thioguanine
THB	trihydroxybutyl
THB-Gua	N7-(2,3,4-trihydroxybutyl)guanine
THB-Val	*N*-(2,3,4-trihydroxybutyl)valine

ALTERNATE TERMS FOR BD, METABOLITES, AND ADDUCTS

BD	α,γ-butadiene
	biethylene
	bivinyl
	buta-1,3-diene
	divinyl
	erythrene
	pyrrolylene
	vinylethylene
	trans-butadiene
BDO	3,4-epoxy-1-butene (EB)
	BD monoepoxide (BMO)
BDO_2	1,2:3,4-diepoxybutane
	BD diepoxide (BDE)
	diepoxybutane (DEB)
BD-diol	3,4-dihydroxy-1-butene
BDO-diol	3,4-epoxi-1,2-butane diol (EBD)
	3,4-epoxy-1,2-butanediol (EBD)
	BD diol-epoxide
	BD monoepoxide-diol
HB-Val	total of 1- and 2-monohydroxy-3-butenyl valine
M1	1,2-dihydroxybutyl mercapturic acid (DHB-MA)
	N-acetyl-*S*-(3,4-dihydroxybutyl)-L-cysteine
M2	1,2-hydroxybutyl mercapturic acid (MHB-MA)
	1-(*N*-acetylcysteinyl)-2-hydroxy-butene
	an isomeric mixture of the regio- and stereoisomers (*R*)/(*S*)-*N*-acetyl-*S*-(1-[hydroxymethyl]-2-propenyl)-L-cysteine + (*R*)/(*S*)-*N*-acetyl-*S*-(2-hydroxy-3-butenyl)-L-cysteine
	monohydroxy-3-butenyl mercapturic acid (MHB-MA)
	total 1- and 2-hydroxy-3-butenyl mercapturic acid (MHB-MA)

Research Report 144, *Genotoxicity of 1,3-Butadiene and Its Epoxy Intermediates,* V. Walker et al.

INTRODUCTION

1,3-Butadiene (BD*; chemical formula CH_2=CH–CH=CH_2) is used extensively in manufacturing synthetic rubber and thermoplastic resins. It is also found in motor-vehicle exhaust and in cigarette smoke. BD is listed by the U.S. Environmental Protection Agency (U.S. EPA) as a mobile-source air toxic and was recently classified as a human carcinogen when inhaled (U.S. EPA 2002a). The U.S. EPA's conclusion is based on a study of workers exposed occupationally in the BD rubber industry that found a greater incidence of certain leukemias compared with control populations (see Delzell et al. 2001).

In the early 1990s, BD was classified as a probable human carcinogen, because the available epidemiologic data were inconsistent. Chronic-inhalation studies in rodents have provided additional evidence for carcinogenicity of BD in rodents (U.S. EPA 2002b), but differences in its mutagenic potency in mice compared with rats complicated extrapolation to humans for the purpose of assessing risk. In the past decade, toxicologic research has therefore focused on assessing the mutagenicity of BD metabolites in mice and rats, and on the difference in BD metabolism between rodent species as it might relate to humans. The major metabolites studied are the reactive monoepoxide 1,2-epoxy-3-butene (BDO); the diepoxide 1,2,3,4-diepoxybutane (BDO_2); and the epoxydiol 1,2-dihydroxy-3,4-epoxybutane (BDO-diol) (Critique Figure 1).

In 1993, HEI issued Request for Applications 93-1, "Novel Approaches to Extrapolation of Health Effects for Mobile Source Air Pollutants". One of the eight studies funded through this RFA was Dr. Walker's "In Vivo Mutation of the Endogenous *Hprt* Genes of Mice and Rats by 1,3-Butadiene and Its Metabolites" (Walker and Meng 2000). In that study, Dr. Walker established the feasibility of examining BD-induced mutations at the *Hprt* gene in T cells of rodents exposed to BD by inhalation. The results showed that the epoxide metabolites BDO and BDO_2 induced *Hprt* mutations, which suggested that they might contribute to the mutagenicity of BD at low levels of exposure (Walker and Meng 2000), whereas other pathways may be activated at higher levels of exposure. Because these metabolites are both stereoisomeric (see sidebar Glossary for Stereochemistry), in its Commentary on that study the HEI Health Review Committee recommended further research to determine which stereoisomeric forms of the metabolites predominate in the tissues of BD-exposed rodents and to evaluate the mutagenicity and mutation spectra of each form.

Dr. Walker submitted a preliminary application for a follow-up study in September 1998. The HEI Health Research Committee was interested in the proposal and asked Dr. Walker to submit a full application, which was then reviewed by external experts and discussed by the committee. Dr. Walker proposed these goals:

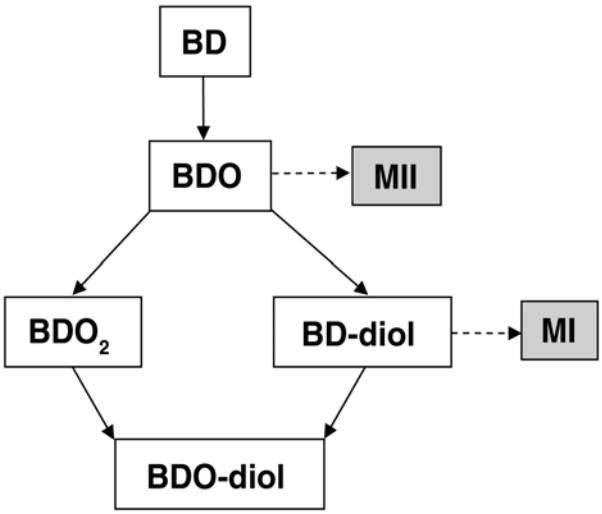

Figure 1. Simplified diagram of BD metabolism. The metabolites BDO, BDO_2, BD-diol, and BDO-diol can all be detoxified and passed from the body. M1 and M2 are two mercapturic acid metabolites that are excreted in urine. The reactive epoxides (BDO, BDO_2, and BDO-diol) can form DNA adducts, which lead to mutations. (See Figure 1 in the Investigators' Report for more details.)

Dr. Walker's 3-year study, "Genotoxicity of 1,3-Butadiene and Its Epoxy Intermediates in Mice and Rats," began in October 1999. Total expenditures were $477,392. The draft Investigators' Report from Walker and colleagues was received for review in July 2003. A revised report, received in July 2004, was accepted for publication in October 2004. 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 Critique.

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

1. Determine the relative mutagenic potencies of the diastereomeric forms of BDO_2 (*meso* and racemic [±] forms) in mice and rats. Earlier studies had used commercially available BDO_2, which did not contain *meso*-BDO_2; Walker proposed synthesizing *meso*-BDO_2 in his laboratory.
2. Develop techniques for measuring BD-diol in blood and tissue and for determining the relative contribution of BD-diol to BD-induced mutagenicity in rats and mice. Not much is known about the role of BD-diol, partly because a method sensitive enough to reliably measure this metabolite at low concentrations had not been developed.
3. Determine the mutagenic potency and specificity of BD in mice and rats exposed to high and low levels of BD. Results from earlier studies had suggested that the metabolism of BD might change with its concentration and that, depending on the exposure level, different metabolites might be responsible for observed mutations. In addition, most studies had been performed in mice, and new data obtained in rats and mice of both sexes would be helpful in understanding sex- and species-related differences in BD mutagenicity.

The HEI Health Research Committee recommended the study for funding because it would address many outstanding questions about the mutagenicity of BD and its metabolites, which would be important in extrapolating results from animal studies to humans.

REGULATORY AND SCIENTIFIC BACKGROUND

BD is part of a large group of toxic and potentially toxic compounds (commonly referred to as air toxics) that are emitted from a wide variety of mobile, stationary, and area sources. In the United States, air toxics are subject to regulations, specified in the Clean Air Act Amendments of 1990, which require that public exposures to cancer-causing hazardous air pollutants be reduced by 75%; the target date for these reductions is 2010. Section 112 of the Amendments lists BD as one of 188 chemicals designated as hazardous. In 1999, the U.S. EPA combined several regulatory efforts required by the Clean Air Act into an Integrated Urban Air Toxics Strategy (U.S. EPA 1999). To address toxic emissions from all outdoor sources, the strategy (1) designates 33 high-priority hazardous air pollutants that are believed to pose the greatest risk to human health; (2) outlines regulatory plans for reducing emissions of mobile-source air toxics; and (3) suggests actions for developing better information on health risks. In 2001, 21 pollutants (including acetaldehyde, benzene, BD, formaldehyde, lead compounds, and polycyclic organic matter) were targeted specifically as mobile-source air toxics emitted by highway vehicles and nonroad equipment (U.S. EPA 2001).

Until the 1980s, scientists considered BD to be relatively nontoxic. Early studies of BD toxicity focused on the irritant effects of short-term, high-level exposures in the workplace, where the permissible exposure limit was set at 1000 ppm as an 8-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 to be a human carcinogen. Early animal studies showed that inhalation of 1000 ppm BD produced tumors in several organs in Sprague-Dawley rats (International Institute of Synthetic Rubber Producers 1981). Later studies showed that mice are much more sensitive to BD than rats (National Toxicology Program 1984, 1992): Inhalation of 625 ppm BD produced

GLOSSARY FOR STEREOCHEMISTRY

Stereochemistry The study of spatial arrangements of atoms in molecules and complexes

Stereoisomers Compounds that have the same constitution and bonding of atoms but that differ in their atomic spatial arrangement

Diastereoisomers Stereoisomeric structures that are not mirror images of one another

Enantiomers Stereoisomeric structures that are mirror images of one another

Racemic mixture A mixture of dextrorotatory (+) and levorotatory (–) optically active isomers; racemic (±) mixtures are not optically active because the optical activities of the enantiomers cancel each other out

S* and *R The possible configurations around a tetrahedral stereocenter (an atom with four bonds to other atoms)

meso A compound that is achiral (i.e., identical to its mirror image) and is therefore not optically active; see also Investigators' Report, Figure 2

Epoxide A ring-shaped organic compound consisting of an oxygen atom bonded to two other atoms, usually carbon, that are bonded to each other; the ring structure is approximately an equilateral triangle, which puts it under strain. The strain makes epoxides more reactive because the ring structure is likely to break, allowing bonds to form with other compounds (e.g., DNA adducts)

Diepoxide A compound containing two epoxide structures

tumors in several organs in mice after 13 weeks of exposure, and exposure to 20 ppm or 6.25 ppm produced rare malignant tumors of the heart and lung, respectively.

Early epidemiologic studies indicated that occupational exposure of workers to BD is associated with an increased incidence of lymphohematopoietic cancers—those of the lymphatic system as well as organs and systems that produce blood cells (Himmelstein et al. 1997; International Agency for Research on Cancer 1999). Possible increases in these two types of cancer were noted in workers exposed to BD in two styrene-BD rubber (SBR) plants (Meinhardt et al. 1982; Santos-Burgoa et al. 1992); but there remained a possibility that exposure to other chemicals in the workplace was responsible for the increased risk. More recent analyses found an increased risk of chronic leukemia in long-term workers in the SBR industry compared with expected rates of leukemia in a matching general population (Delzell et al. 2001). Furthermore, an increased rate of lymphosarcoma, but no excess risk of chronic leukemia, was observed in longitudinal studies of workers in BD-monomer production who experienced short-term, high-level exposures associated with wartime production before 1950 (Divine and Hartman 1996).

The apparent discrepancies among these studies might be caused by the low concentrations of BD and a smaller number of workers evaluated in the BD-monomer plants or by the presence of styrene or some other confounding substance (such as the stopping agent dimethyldithiocarbamate [DMDTC]) in the SBR production facilities. A recent study has extended and reevaluated the largest cohort of exposed workers in the SBR industry (Delzell et al. 2006). That study has contributed new results that strengthen the epidemiologic evidence concerning the carcinogenicity of BD. In particular, the analyses indicated that (1) occupational exposure to BD might increase the risk of lymphohematopoietic cancers, and leukemia in particular; (2) the excess relative risk observed in workers in the SBR industry (compared with risks in the general population) increases as a function of cumulative exposure; and (3) these results persist when workplace exposures to other putative carcinogens, such as styrene and DMDTC, are accounted for (Delzell et al. 2006).

The U.S. EPA has conducted several quantitative risk assessments of BD since the mid-1980s. Its first estimate, based on tumor data from mice, indicated that for every 1000 people in the general population, 250 cancer deaths could be attributed to the BD exposure when a lifetime exposure to 1 ppm BD is assumed (U.S. EPA 1985). In 1998, the U.S. EPA reported a much lower risk estimate of 9 cancer deaths for every 1000 people (U.S. EPA 1998). This assessment used data from occupationally exposed workers in the SBR industry and retrospective exposure estimates developed from work-history records (Delzell et al. 2001). In its most recent risk estimate, the U.S. EPA applied the relative risk of leukemia for male synthetic rubber workers to incidence rates for leukemia in the U.S. and obtained a lifetime leukemia incidence risk of 8 per 1000 exposed workers (U.S. EPA 2002a).

Because of uncertainties in understanding the risk of cancer in exposed workers, human risk assessments have generally relied on data from animal studies. Such estimates have been problematic, however, because of the uncertainties in extrapolating results from rodents to humans, and because the carcinogenic potency of BD varies among species, possibly because of differences in the production of various BD metabolites. The marked difference in sensitivity between mice and rats raises the question of which species most closely resembles humans in its response to BD. Research into the mechanisms of carcinogenicity in the two rodent species has included efforts to determine the sequence of metabolic reactions that induce cancer and to identify the critical reactions that might explain the species-related differences in susceptibility. Better understanding of the reasons for the species' differences will increase our understanding of human sensitivity to BD exposure.

When BD is metabolized, it is either converted to reactive epoxy intermediates (which are potentially carcinogenic) or detoxified to water-soluble metabolites (which are readily excreted from the body). In all species that have been studied, a common metabolic pathway has been identified (Critique Figure 1; details appear in Figure 1 of the Investigators' Report). BD is first metabolized by cytochrome P450-monooxygenase to the reactive monoepoxide BDO. BDO can then be metabolized further to either 1,2-dihydroxy-3-butene (BD-diol) or the reactive diepoxide BDO_2. BD-diol and BDO_2 can both be oxidized to the reactive epoxydiol BDO-diol. These metabolic steps are regulated by two different enzymes, cytochrome P450-monooxygenase (which aids in the formation of epoxides) and epoxide hydrolase (which aids in the formation of alcohols from epoxides). The epoxides BDO, BDO_2, and BDO-diol can (1) react with macromolecules such as DNA or proteins, or (2) be detoxified by glutathione *S*-transferase to a glutathione conjugate, which is excreted in the urine.

Although BD is converted to the same metabolites in rats and mice, important differences between the species have been noted in the proportions of the metabolites found in the body. There might be differences in how BD and its metabolites are absorbed, distributed, transformed, and excreted over time and in which biological processes they participate. Research with rats and mice has been

directed toward (1) identifying the BD metabolites that contribute to carcinogenesis, (2) quantifying the proportion of each metabolite's presence, and (3) calculating whether species-related differences in the rates at which epoxide metabolites are produced and detoxified contribute to the observed differences in cancer incidence between the two species (Himmelstein et al. 1997; Richardson et al. 1999).

In the past decade, HEI's BD research program has included several animal and human studies that have yielded important knowledge about species-related differences in the mutagenic and carcinogenic effects of BD and its metabolites in rodents (Henderson et al. 2000; Recio et al. 2000; Walker and Meng 2000), and about the formation of DNA and hemoglobin adducts and their correlation with BD exposures in rodents and humans (Albertini et al. 2003; Blair et al. 2000; Swenberg et al. 2000). In addition, HEI funded a follow-up study of the workers in the SBR industry (Delzell et al. 2006). After completion of the animal studies, questions remained about species- and sex-related differences in BD metabolic pathways, specifically at low exposure concentrations, and about the role of the stereochemistry of epoxy metabolites in their mutagenicity. Dr. Walker sought to address the questions about low-level exposures and sex-related issues in the follow-up investigation reported here. A study by Dr. Meng and colleagues, funded under HEI's Walter A. Rosenblith New Investigator Award (Meng et al. 2009), has systematically investigated the effects of stereochemistry for all of the BD epoxy metabolites.

SPECIFIC AIMS

Dr. Walker addressed the following specific aims in his investigation:

1. To determine *Hprt* mutant frequencies in female mice exposed to a low level of BD via inhalation, and to further define the shape of the exposure–response curve for mutant frequencies over a wide range of BD concentrations (3 to 1250 ppm);
2. To assess the influence of age and sex of mice and rats on mutagenic responses to a high-level exposure to BD (1250 ppm);
3. To determine in mice and rats the mutagenic potency of *meso*-BDO_2 compared with the racemic mixture (±)-BDO_2;
4. To develop techniques for measuring BD-diol in plasma and to determine the relative contribution of BD-diol and its metabolite BDO-diol to BD-induced mutagenicity; and
5. To develop a multiplex PCR procedure to detect *Hprt* mutations and to use it in combination with reverse transcription–polymerase chain reaction (RT–PCR) to identify both small- and large-scale genetic changes across the *Hprt* locus.

APPROACH

The investigators conducted experiments with male and female F344 rats and B6C3F1 mice; animals were 4 to 5 weeks old at exposure except the female mice for Specific Aim 2, which were 8 to 9 weeks old. Animals were exposed via inhalation to BD or its metabolites BDO_2 and BD-diol for 6 hours/day, 5 days/week. Control groups were exposed to filtered air. Upon completion of the exposures, lymphocytes were collected from the spleen for determination of *Hprt* mutations (see sidebar *Hprt* Mutation Assay). Some target and nontarget tissues were stored for future studies of DNA and hemoglobin adducts. For the BD-diol experiments, plasma and urine were analyzed for concentrations of BD-diol and other metabolites. A detailed overview of the exposure groups is provided in Table 1 of the Investigators' Report; a brief overview is provided here (hereafter, all figure and table numbers refer to the Investigators' Report unless otherwise stated).

BD EXPOSURE–RESPONSE (SPECIFIC AIM 1)

For Specific Aim 1, *Hprt* mutant frequencies were determined in 4- to 5-week-old female mice exposed for 2 weeks to a low level of BD (3 ppm). These data were combined with data from earlier studies to expand the exposure–response curve for BD.

AGE- AND SEX-RELATED EFFECTS AND MOLECULAR ANALYSES OF MUTATIONS (SPECIFIC AIMS 2 AND 5)

For Specific Aim 2, *Hprt* mutant frequencies were assessed in male rats and in male and female mice exposed for 2 weeks to a high level of BD (1250 ppm) to evaluate age- and sex-related differences in mutagenic responses to BD. The males were 4 to 5 weeks old, and the female mice were 8 to 9 weeks old. These data were compared with data obtained from earlier studies (Walker and Meng 2000) in which 4- to 5-week-old female mice and rats had been exposed to BD.

For Specific Aim 5, cells derived from the male mice and rats exposed to 1250 ppm BD for Specific Aim 2 were cloned and propagated for molecular analysis of clones containing presumptive mutations.

BDO_2 STEREOCHEMISTRY (SPECIFIC AIM 3)

Female mice and rats (4 to 5 weeks old) were exposed for 3 weeks to 2 ppm *meso*-BDO_2 and killed at different times after exposure to assess the time course of *Hprt* mutant frequencies. These data were compared with data from female mice and rats exposed to 2 ppm (±)-BDO_2 in earlier studies (Walker and Meng 2000).

In addition, 4- to 5-week-old mice and rats of both sexes were exposed for 2 weeks to 2 or 4 ppm *meso*-BDO_2 to evaluate sex-related differences. Cells derived from males exposed to 2 or 4 ppm *meso*-BDO_2 were cloned and saved for future analyses of mutation spectra.

BD-DIOL EXPERIMENTS (SPECIFIC AIM 4)

The investigators first developed a sensitive method for analyzing concentrations of BD-diol in plasma (see the section Measurement of BD-diol in Plasma and Urine below).

Male and female mice and rats were then exposed nose-only to BD (0, 62.5, 625, or 1250 ppm) or BD-diol (0, 6 or 18 ppm) for 6 hours to determine the BD-diol exposure level that would yield BD-diol plasma concentrations similar to those produced by exposure to 62.5 and 625 ppm BD. (These exposures are shown as Inhalation Studies A and B in Table 1.)

Next, male and female mice and rats were exposed to 0, 6, or 18 ppm BD-diol, and male and female mice and female rats were exposed to 0 or 36 ppm BD-diol for 4 weeks in an inhalation chamber and killed at different times after exposure to determine the time course and extent of *Hprt* mutant frequencies. Cells derived from male animals were cloned and saved for future analyses of mutation spectra. (These exposures are shown as Inhalation Studies C and F in Table 1.)

In addition, female rats and mice were exposed nose-only to BD (0 or 200 ppm) or BD-diol (0, 24, or 36 ppm) for 6 hours to provide additional data on concentrations of BD-diol in plasma and of the two mercapturic-acid metabolites, M1 and M2 (Critique Figure 1), in urine. (These exposures are shown as Inhalation Studies D and E in Table 1.)

BRIEF OVERVIEW OF METHODS

ANIMAL EXPOSURES

Most exposures were conducted for 2, 3, or 4 weeks in whole-body chambers, to which the animals had been conditioned for 2 days before the exposure period. Exposures were 6 hours/day, 5 days/week plus the time it took to reach 90% of the target concentration in the chamber. The single 6-hour exposures under Specific Aim 4 were nose-only.

BD exposure atmospheres were monitored periodically in real time using an infrared spectrometer; in addition, air samples were collected three times each day and analyzed by gas chromatography (GC) and a flame ionization detector. BDO_2 atmospheres were monitored by collecting samples for 60 minutes, using glass impingers containing

HPRT MUTATION ASSAY

The *Hprt* assay used by Dr. Walker assesses gene mutations in mammalian cells in vitro to examine genetic damage at one gene that might reflect genetic damage throughout the genome. The *Hprt* gene codes for the enzyme hypoxanthine guanine phosphoribosyl transferase, which aids cells in using purines to build nucleotides for DNA synthesis. It is not essential for synthesizing new DNA, meaning that cells can survive without it. If the enzyme encounters a toxic purine analog, such as 6-thioguanine (6-TG), the analog is converted to a nucleotide derivative and incorporated into newly synthesized DNA, resulting in cell death. Mutations to the *Hprt* gene disable the enzyme and the conversion of purines, allowing mutant cells to survive and to continue to divide. Thus, cells *without* a mutation in *Hprt* die when exposed to 6-TG; only cells *with* mutations in *Hprt* form visible colonies when exposed to 6-TG.

Mutations can occur spontaneously or when cells are exposed to a mutagenic compound. Because not every cell forms a colony, **cloning efficiency** expresses the percentage of cells that form colonies under normal conditions (i.e., in the absence of 6-TG). To determine cloning efficiency, wells are seeded with a small number of cells (4 or 8) in the presence of thousands of lethally irradiated "feeder cells." Wells with and without colonies are counted after enough time has passed for colony formation (usually 1 to 3 weeks, depending on the growth rate of the cells).

Mutant frequency expresses the number of cells that are mutants, which is detected by their ability to grow in the presence of 6-TG. Since mutant cells also form colonies at less-than-perfect efficiency, cloning efficiency is taken into account when calculating mutant frequency. Typically, wells are seeded with several thousand cells, and the number of wells containing mutant colonies is determined after enough time has passed for colony formation. Cloning efficiency and mutant frequency are always determined in parallel.

ethyl acetate, and immediately analyzing them by GC/mass spectrometry. BD-diol atmospheres were monitored in real time using a flame ionization detector; in addition, integrated samples were collected for 30 minutes, using glass impingers containing purified water, and analyzing them using GC/flame ionization detection.

ASSESSMENT OF MUTANT FREQUENCIES

Upon completion of the exposures, animals were killed and their spleens removed for isolation of lymphocytes. The spleens were mashed to generate a single-cell suspension of lymphocytes, which were then activated and grown in culture medium. The lymphocytes were grown in the presence or absence of 6-thioguanine to determine the mutant frequency in the *Hprt* gene (see sidebar *Hprt* Mutation Assay).

MEASUREMENT OF BD-DIOL IN PLASMA AND URINE

The investigators developed a sensitive method of measuring BD-diol in plasma and urine based on a butylboronate-derivatization procedure described by Giachetti and colleagues (1989). They used human urine and plasma when developing methods because human samples were readily available under an existing protocol approved by the Institutional Review Board; this reduced the need to purchase and kill additional animals.

Blood samples were collected at different times after exposure. Upon completion of the exposures, some female mice and rats were placed in metabolic cages for 24 hours to collect urine samples.

ASSESSMENT OF MUTATION SPECTRA

Mutation spectra can provide insight into how compounds differ in causing various types of mutations and can help identify the location of the mutations and their frequency. Examples of different types of mutations are deletions and insertions of base pairs and substitutions of a single base. Deletions and insertions may lead to frameshifts; because amino acids are coded by three base pairs in the genome, adding or deleting one or more base pairs causes a shift in the way the genome is decoded, which leads to an altogether different sequence of amino acids in the expressed protein. Single-base substitutions (or point mutations) can be transversions (in which a purine base [A or G] replaces a pyrimidine base [C or T] or vice versa) or transitions (in which a purine replaces another purine or a pyrimidine replaces another pyrimidine). Point mutations may or may not lead to a different amino acid depending on which base substitution occurs.

In this investigation, mutation spectra were assessed across the entire *Hprt* gene, in both messenger RNA and DNA. Clones containing mutations were lysed and prepared for RT–PCR analysis of total RNA. Using rat and mouse *Hprt*-specific primer, complementary DNA was formed, multiplied, and sequenced. This method detects base substitutions and frame shifts.

A second method used multiplex PCR to determine mutations in genomic DNA extracted from mutant clones using a DNA isolation kit. After all nine *Hprt* exons (the regions of the gene that code for proteins) were amplified, mutations were analyzed using specific primers. Amplification of exon 2 of the K-*ras* gene served as a positive control. The amplification products were separated by electrophoresis and stained with ethidium bromide for visualization under UV light. This method detects large deletions of one or more exons.

STATISTICAL ANALYSES

The main analyses compared cloning efficiencies and mutant frequencies of exposed rodents with those of control animals. The Mann-Whitney Rank Sum test was used to determine significant differences at each time point. Bonferroni adjustments of the *P* values were made if a significant difference was observed at more than one time point. In addition, the investigators developed a method to estimate the overall mutagenic potency (taking into account the mutant frequencies at different time points) by fitting a polynomial model to the temporal mutant-frequency data and repeating the curve-fitting steps over 5000 simulations. Using this resampling or bootstrapping method, a 95% confidence interval was estimated from the 2.5 and 97.5 percentiles of the potency estimates.

Significant differences in mutation spectra were assessed using the generalization of the Fisher exact test to contingency tables, which investigated differences in the proportion of individual types of mutations. Power calculations were performed to estimate the sample sizes required to detect a significant mutagenic effect.

SUMMARY OF RESULTS

BD EXPOSURES (SPECIFIC AIMS 1 AND 2)

The investigators used data from previous experiments performed under identical conditions for comparison (Walker and Meng 2000); they found that females were more sensitive to the mutagenic effects of BD exposure than males and that mice were more sensitive than rats (Figure 7). Female mice were the most sensitive; mutagenicity was observed at even the lowest concentration of 3 ppm BD

(Figure 4). Female mice that were 4 to 5 weeks old showed higher mutant frequencies after exposure to 1250 ppm BD than did 8- to 9-week-old female mice (Figure 6), which indicates that age-related differences in the activity of the thymus and in T-cell trafficking play a role.

meso-BDO_2 EXPOSURES (SPECIFIC AIM 3)

The results obtained after exposure to *meso*-BDO_2 were compared with results from earlier studies in which rats and mice had been exposed to the racemic (±) form of BDO_2 using identical experimental protocols (Walker and Meng 2000). There was little difference in the mutagenic properties between the racemic and *meso* forms of BDO_2 (Table 5); this indicates that the stereochemistry of BDO_2 did not play a role in its mutagenicity. After exposure to *meso*-BDO_2, higher mutant frequencies were observed in rats than in mice, as had been the case with (±)-BDO_2 in the previous studies. These results indicate that when BDO_2 is compared with BDO and BD-diol, its overall mutagenic potency is more important than the relative potency of any of its isomers in explaining differences in species' responses. In the future, complete characterization of BDO_2-derived DNA adducts may provide supporting evidence and help place these results in greater perspective.

BD-DIOL EXPOSURES (SPECIFIC AIM 4)

Using the newly developed method to measure BD-diol in plasma, the investigators showed that for mice, exposure to 18 ppm BD-diol or to 200 ppm BD produced similar concentrations of BD-diol in plasma (Inhalation Studies B, C, and D; Figure 15).

For female rats, exposure to 625 ppm BD or to 36 ppm BD-diol resulted in the same mutant frequency (Inhalation Studies A and E; Table 5), indicating that the contribution of the BD-diol–derived metabolites to the mutagenicity of BD is greatest at high BD exposure levels. Thus, at high BD concentrations, BDO-diol derived from BD-diol may be largely responsible for the mutagenic effects observed.

MUTATION SPECTRA (SPECIFIC AIM 5)

Analysis of a number of mutations in isolated clones of *Hprt* mutants in male rats and mice indicated that both small- and large-scale deletions were commonly observed after BD exposure. There were species-related differences in which types of lesions were observed, including a higher frequency of G•C → C•G substitutions and frameshifts in mice and a higher frequency of A•T → G•C substitutions and complex mutations in rats (Table 12). With the methods developed in this study, future analyses of mutation lesions from animals exposed to BDO_2 or BD-diol should provide more insight into the role of these metabolites.

DISCUSSION

This investigation was carefully designed and conducted. It yielded important new information on the metabolism and mutagenicity of BD and its metabolites in mice and rats. The range of exposure concentrations was one strength of the study, as were the comparisons between species, age groups, and sexes. The findings at low exposure concentrations (3 ppm) are especially important, because they approach occupational exposure levels.

Dr. Walker and colleagues provide a range of data that complement earlier studies on BD mutagenicity in mice and rats exposed to BD, BDO, and BDO_2. The present study provides more information on BD's higher mutagenicity in mice than in rats. The finding that female mice are the most sensitive of the rodents examined confirms results from earlier studies (Walker and Meng 2000). Future research might investigate even lower exposures in female mice to assess the lower bounds of the BD exposure–response curve. In addition, the current study contained power calculations to estimate the sample sizes that will be required to detect a significant mutagenic effect in future studies (Table 3).

Using data from this and earlier studies (Walker and Meng 2000), the investigators compared the mutagenic potency of BD, BDO, BDO_2, and BD-diol in mice and rats (Table 5). They found that rats had higher mutant frequencies than mice when exposed to the same concentration of BDO_2. This is unexpected because earlier studies have shown that mice had higher blood concentrations of BDO_2 than rats when both were exposed to the same concentration of BDO_2. In fact, this difference in blood concentrations has been suggested as the main reason for the higher rate of tumor formation in mice than in rats (Henderson et al. 1999).

Dr. Walker mentioned two possible explanations. First, the efficiency of hepatic clearance of BDO_2 by hydrolysis is lower in rats than in mice (Boogaard and Bond 1996). Second, rat lymphocytes appear to tolerate the toxic effects of BDO_2 better than mouse lymphocytes do (Henderson et al. 1999); higher lymphocyte survival rates in rats might contribute to the higher mutant frequencies in rats compared with those in mice. It is also possible that mice and rats have different rates of DNA repair; whether this is true remains to be determined. Furthermore, the investigators found that the stereochemistry of BDO_2 did not seem to play a role in its mutagenicity. (Dr. Meng, with funding from HEI, has been investigating the roles that the nine stereoisomers of the three epoxy metabolites have in BD mutagenicity in the TK6 human lymphoblastoid cell line. The final report of this study is currently in press.)

Dr. Walker and colleagues also provided novel data on the metabolic pathway involving BD-diol and BDO-diol. Previously, the role of BD-diol could not be assessed in detail because existing assays were not sensitive enough to detect it. Originally, Dr. Walker had proposed to develop an assay to measure BD-diol and evaluate its plasma concentrations in rodents exposed to BD. Instead, he opted for the clever approach of exposing rodents to BD-diol directly. Thus, specific stereoisomers of BD-diol's metabolite BDO-diol were formed in vivo without the formation of the other epoxide metabolites (BDO and BDO_2).

The investigators found that at high BD exposure concentrations (625 ppm), BD-diol–derived BDO-diol might be largely responsible for the mutagenic effects observed in both rats and mice. In mice, BDO_2 also contributes to mutations at high BD concentrations and BDO plays a minor role. At low BD exposure concentrations (< 62.5 ppm), BDO_2 is largely responsible for mutations observed in mice but not in rats and BDO does not seem to play a role at these concentrations.

In vitro, BDO_2 is a much more potent mutagen than the other epoxides. The observation that mice have considerably higher concentrations of BDO_2 in blood and tissue after BD exposure (Thornton-Manning et al. 1995) has been proposed as the main reason why mice have a higher rate of tumor formation. The current finding that exposure to equal concentrations of BDO_2 caused a higher mutant frequency in rats than in mice might seem contradictory. However, slightly higher mutant frequencies might not translate into higher rates of tumor formation because the BDO_2 pathway does not play a large metabolic role in rats; this is evidenced by the fact that BDO_2 concentrations in plasma and tissue are much lower in rats than in mice after BD exposure (Thornton-Manning et al. 1995).

PURITY OF CHEMICALS

One important issue in this and other studies that investigate the effects of exposure to chemicals is the purity of the chemical compound. Because potentially toxic impurities would be important confounders, it is of paramount importance that investigators do not rely on a manufacturer's assurance of the purity of chemicals but thoroughly analyze the actual purity of the compound being used. The investigators did include manufacturer-provided purity data in the report but did not perform their own chemical analyses. Therefore, some uncertainty remains about the results. The Health Review Committee recommends that investigators perform detailed chemical analyses of the purity of the compounds under investigation in future studies.

MUTATION SPECTRA

Analysis of a number of mutation lesions in isolated clones of *Hprt* mutants after exposure to BD showed that both small and large deletions were common. This reflects the improved methods used, which allowed the investigators to detect lesions that had been difficult to identify. However, analysis of much larger numbers of mutants will be required to firmly establish the role of BD and its metabolites in deletions at the *Hprt* gene. Dr. Walker has collected samples for future analysis of mutation spectra after exposure to BDO_2 and BD-diol to determine the role of these metabolites in BD-induced mutagenicity. Further analysis of mutations and DNA adduct formation might also be interesting—to study specific point mutations, for example, or to investigate whether adducts occur in tumor suppressor genes.

Future studies should focus on a comprehensive analysis of metabolite concentrations (including identification of stereoisomeric compositions), kinetics, and adduct levels, coupled with in vivo analysis of mutant frequencies. Such a comprehensive approach could provide the information needed to determine which metabolites are critical to the toxicity of BD and how best to extrapolate from rodents to humans, especially at low levels of exposure (3 ppm and below).

STATISTICAL ANALYSES

The investigators used a novel statistical approach to analyze the mutagenic-potency data obtained at different nonlinear time points. In the bootstrap analysis, simulations were run to derive confidence limits around the time–response curve. Because this is a complicated analysis that might have underestimated the variability of the data, the Health Review Committee recommends using a different sampling approach to estimate confidence intervals. However, the authors should be commended for trying a novel approach. Ultimately, the conclusions drawn by the investigators were probably not affected.

Simulation-based methods were used in several analyses, involving several assumptions about key features of the simulation process. Little information about these assumptions is provided in the report. Furthermore, in most experiments, measurements were taken at a number of time points or a number of exposure levels. Typically, comparisons were made using two-sample tests at each time point or each exposure level. A combined analysis would have been more appropriate and efficient.

SUMMARY

This investigation has provided important data on the mutagenic potency of BD at low exposure concentrations (3 ppm). In addition, analyses by age and sex confirmed and extended earlier observations on the greater susceptibility of female rodents. The greater susceptibility of mice compared with rats was also reaffirmed. Rodents 4 to 5 weeks old were found to be more sensitive than those 8 to 9 weeks old, which the investigators attributed to differences in thymus activity and movement of T cells through the body. Dr. Walker and colleagues showed that the contribution of the metabolites BD-diol and BDO-diol to BD-induced mutagenicity is most pronounced at high exposure concentrations; the contribution of the metabolite BDO_2 is probably more pronounced at lower concentrations. They found that stereochemistry did not play a role in BDO_2-induced mutagenicity; however, the in vivo role of the stereochemistry of the monoepoxide metabolites BDO and BDO-diol remains to be established.

Future studies should include a comprehensive analysis of metabolite concentrations, kinetics, and adduct levels, coupled with in vivo analysis of mutant frequencies. In addition to knowing each metabolite's mutagenic potency, the process of formation and elimination of metabolites in the body should be carefully assessed, as well as the extent to which metabolites form DNA adducts that may cause mutations or other genetic damage. In the past, several studies have investigated these issues, but mostly in isolation. A comprehensive approach could provide the information needed to determine which metabolites are critical to the toxicity of BD and how best to extrapolate from rodents to humans, especially at low levels of exposure (3 ppm and below).

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