Metabolism of Butadiene Monoxide by Freshly Isolated Hepatocytes From Mice and Rats: Different Partitioning Between Oxidative, Hydrolytic, and Conjugation Pathways

Raymond A. Kemper,1 Renee J. Krause, and Adnan A. Elfarra

Department of Comparative Biosciences and Center for Environmental Toxicology, University of Wisconsin, School of Veterinary Medicine, Madison, Wisconsin

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ABSTRACT:
1,3-Butadiene (BD) is a multisite carcinogen in rodents, with mice being much more susceptible than rats. This species difference in carcinogenicity has been attributed to differences in metabolism. In this study, coordinated metabolism of butadiene monoxide (BMO, 5, 25, and 250 μM), the primary reactive metabolite of BD, was investigated in freshly isolated male B6C3F1 mouse and Sprague-Dawley rat hepatocytes. The hepatocytes from both species catalyzed BMO oxidation to meso- and (±)-diepoxynbutane (DEB), BMO hydrolysis to 3-butene-1,2-diol (BDD), and BMO conjugation with glutathione (GSH) to form GSH conjugates (GSBMO). Metabolite area under the curve (AUC) exhibited dependence on the BMO concentration and incubation time (0–45 min). However, the observed BMO activation/detoxication ratios (obtained by dividing the AUC for total DEB by the summed AUC values for BDD and GSBMO) with mouse hepatocytes were approximately 15- to 40-fold higher than the corresponding ratios observed with rat hepatocytes. At 5 μM BMO, bioactivation in the mouse exceeded detoxication by approximately 2-fold, whereas at the 250 μM concentration, activation was only about 31% of total detoxication. In rat hepatocytes, the activation-detoxication ratio was relatively independent of the initial BMO concentration, with flux through the oxidative pathway at approximately 2 to 5% of the total detoxica- tion. These results, which are more consistent with in vivo mouse and rat toxicity data than the metabolic rates obtained with subcellular fractions, illustrate the potential utility of the isolated hepatocyte model for estimating flux through competing metabolic pathways and predicting in vivo metabolism of BMO and its parent compound, BD.

Butadiene (BD2) is a high production commodity chemical used extensively in the manufacture of synthetic rubber and thermoplastic resins. In addition, BD is found in cigarette smoke, automobile exhaust, and at low concentrations in urban air (Melnick and Kohn, 1995). BD is mutagenic in mammalian and bacterial assays in the presence of an S-9 activating system (Duvurger et al., 1981; Tice et al., 1987) and is carcinogenic in mice and rats following long-term inhalation exposure (Owen et al., 1987; Melnick et al., 1990). Epidemiological evidence suggests a link between occupational exposure to BD and an increased risk of hematopoietic cancers (Delzell et al., 1996; Divine and Hartman, 1996). The International Agency for Research on Cancer (IARC) (1999) has classified BD as a probable human carcinogen, whereas the United States Department of Health and Human Services National Toxicology Program (2000) has recently upgraded BD to the classification of “Known to be a Human Carcinogen”. Mice are significantly more sensitive to the carcinogenic activity of BD compared with rats, and this difference in susceptibility is thought to be related in large part to differences in bioactivation of BD to reactive epoxide metabolites.

Oxidation of BD by cytochrome P450s results in the formation of butadiene monoxide (BMO; Fig. 1). This reaction has been demonstrated in microsomes from several rodent and human tissues; the primary P450s involved in BD oxidation in human liver are CYP2E1 and CYP2A6 (Malvoisin et al., 1979; Csanady et al., 1992; Duescher and Elfarra, 1994). BMO forms covalent adducts with DNA (Citti et al., 1984; Tretyakova et al., 1997; Selzer and Elfarra, 1999), and it is directly mutagenic in bacterial and mammalian assays (de Meester et al., 1984; Melnick and Kohn, 1995). For this reason, BMO research has been a major focus in BD metabolism studies. However, the primary pathway for BD detoxication involves conjugation with GSH (Sharer et al., 1991, 1992), which are subsequently eliminated in the urine (Sharer et al., 1991, 1992), and the resulting conjugates are excreted into the bile (Sharer and Elfarra, 1992) or metabolized further to mercapturic acids, which are subsequently eliminated in the urine (Sharer et al., 1991, 1992). The second pathway for BMO metabolism involves P450-mediated oxidation, resulting in formation of diepoxynbutane (DEB). This reaction has been demonstrated in mouse, rat, and human liver micro-
somess (Seaton et al., 1995; Krause and Elfarra, 1997), and it leads to formation of meso- and (±)-DEB (Krause and Elfarra, 1997). Human liver microsomal oxidation of BMO to DEB is carried out primarily by P450 2E1, but P450s 2A6, 2C9, and 3A4 may also contribute to this pathway, particularly at higher substrate concentrations (Seaton et al., 1995; Krause and Elfarra, 1997). Because the in vitro mutagenic potency of DEB is approximately 100-fold greater than BMO (Chochrome and Skopek, 1994), DEB is thought to play a critical role in the carcinogenic activity of BD. The third mechanism for elimination of BMO is catalyzed by epoxide hydrolases (EH). BMO is hydrolized by microsomal EH in rats, and by both microsomal and soluble forms in mice and humans (Krause et al., 1997), leading to the formation of 3-buten-1,2-diol (BDD). EH-mediated hydrolysis is believed to be a major elimination pathway for BMO in rat and human liver (Betcholt et al., 1994; Krause et al., 1997).

From the preceding discussion, it is apparent that BMO plays a pivotal role in metabolism and toxicity of BD. BMO undergoes bioactivation via oxidation to DEB and is detoxified by conjugation with GSH and by enzymatic hydrolysis. Hence, the carcinogenic activity of BD is likely to be closely linked to the balance between activation and detoxication of BMO. Previous in vitro studies of BMO metabolism have examined bioactivation and detoxication reactions in isolation (Kreuzer et al., 1991; Csanady et al., 1992; Sharer et al., 1995; Seaton et al., 1995; Boogaard et al., 1996; Krause and Elfarra, 1997; Krause et al., 1997), and the kinetic parameters determined from these studies have been compared in an attempt to draw conclusions concerning the balance of activation and detoxication. Such studies have yielded useful enzyme kinetic data. However, since enzyme systems seldom function in isolation, the ability of subcellular metabolic systems to predict coordinated in vivo metabolism is limited. The studies described here examine metabolism of BMO in freshly isolated mouse and rat hepatocytes. Isolated hepatocytes are ideal for investigating metabolism of compounds that are subject to multiple parallel and sequential enzymatic pathways because they allow direct estimation of net exposure of the system to the metabolites of interest. Hepatocytes also retain more of the biological complexity of the whole animal, compared with subcellular fractions (e.g., intracellular compartmentalization of enzymes, physiological cofactor concentrations). The current report compares flux through competing P450-, GST-, and EH-mediated pathways for BMO metabolism in isolated rodent hepatocytes at various initial substrate concentrations. BMO was used as substrate in these studies to facilitate a detailed investigation of its metabolic fate, since this compound represents a critical branch point in the carcinogenic action of BD, and the results obtained may enhance future experimental designs with BD.

**Materials and Methods**

**Chemicals.** Trypsin inhibitor (type II-O) was obtained from Sigma Chemical Co. (St. Louis, MO). BMO, (±)-DEB, butane-1,4-diol, reduced GSH and cyclohexene oxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). (±)-BDD was obtained from Acros Chemicals (Pittsburgh, PA). Collagenase H (from *Clostridium histolyticum*) was obtained from Boehringer Mannheim (Indianapolis, IN). Hanks’ balanced salt solution (HBSS) was obtained from Life Technologies (Gaithersburg, MD). N,O-bis(trimethylsilyl)- trifluoroacetamide + 10% trimethylchlorosilane (BSTFA + 10% TMCS) was obtained from Pierce Chemicals (Rockford, IL). S-(2-Hydroxy-3-buten-1-yl)-glutathione and S-(1-hydroxy-3-buten-2-yl)glutathione, the GSH conjugates of BMO (GBMO), were synthesized and purified as described previously (Sharer et al., 1992). All other chemicals were of reagent grade or higher, and all solvents were of GC or HPLC grade.

**Animals.** Male B6C3F1 mice (8–10 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). Male Sprague-Dawley rats (175–230 g) were obtained from Charles River Laboratories (Raleigh, NC). Animals were maintained on a 12-h light/dark cycle and were allowed food and water ad libitum. Hepatocytes from mice and rats were isolated by two-step EDTA/collagenase perfusion using a modification of the method described by Kedders and Held (1996). Hepatocytes were prepared at approximately the same time of day for all experiments to minimize circadian variation in enzyme levels, GSH, and other cofactors. Briefly, animals were anesthetized deeply and the liver was perfused via the vena cava for the mice or the portal vein for the rat first with calcium-free HBSS containing 0.5 mM EDTA followed by 1 mg/ml Collagenase H in HBSS containing 5 mM CaCl2 and 0.27 mg/ml trypsin inhibitor. Each solution was perfused for approximately 8 min at 5 ml/min for mice and 10 ml/min for the rat. After perfusion, the liver was carefully excised, placed into a weighing boat filled with Krebs-Henseleit buffer and the solution passed through a 292-m nylon mesh. The filtrate was centrifuged again at 50,000 g for 2 min at 50°C. The pellets were resuspended in fresh KH buffer and the solution passed through a 292-μm nylon mesh. The filtrate was centrifuged again at 50,000 g and resuspended in fresh KH buffer. Cell yield and viability were determined by trypsin blue exclusion using a hemocytometer. For metabolic experiments, cells were diluted to a concentration of 1.36 × 10⁶/ml in KH buffer supplemented with 0.1% glucose and 0.2% bovine serum albumin, and gently teased apart with forceps. Dissociated cells were recovered by centrifugation at 4°C for 2 min at 50°C. The pelleted cells were resuspended in fresh KH buffer and the solution passed through a 292-μm nylon mesh. The filtrate was centrifuged again at 50,000 g and resuspended in fresh KH buffer. Cell yield and viability were determined by trypan blue exclusion using a hemocytometer. For metabolic experiments, cells were diluted to a concentration of 1.36 × 10⁶/ml in KH buffer supplemented with 0.1% glucose and 0.2% bovine serum albumin, and maintained on ice until use.

**Metabolic Reactions.** Reactions were carried out in 24-nl vials with screw caps fitted with Teflon-faced septa. Hepatocyte suspension (2.2 ml, 3 × 10⁶ cells) was placed into vials and purged briefly with 95% O₂/5% CO₂ before the vials were sealed. Reactions were carried out at 37°C with gentle shaking (175 rpm). After a 4-min preincubation, the reaction was initiated by addition of 100 μl of BMO solution (in 0.9% NaCl) to give final concentrations of 5, 25, or 250 μM in a volume of 2.3 ml. The BMO solution was introduced through the septum using a gas-tight syringe. At 5-min intervals, reactions were terminated by immersion in an ice bath. For quantitation of meso- and (±)-DEB and BDD, 100 μl of a solution containing 0.5 mM cyclohexeneoxide and 0.5 mM butane-1,4-diol in KH buffer was added after sample cooling on ice (internal standards for DEB and BDD, respectively). After gentle mixing, a 50-μl aliquot of cell suspension was removed and cell viability was determined by trypan blue exclusion. BMO metabolism to GBMO was examined in separate experiments carried out exactly as for BMO metabolism to DEB and BDD, except that the internal standards were omitted.

**Sample Preparation.** Cell suspensions were transferred to test tubes and centrifuged at 1500 g for 5 min at 4°C to sediment the cells. Recovery experiments indicated that both DEB and BDD (10–30 μM) were rapidly and uniformly distributed throughout the cell suspension. For this reason, metabolites were measured in the reaction medium, which produced an extract that was free of significant analytical interferences. An aliquot (1.5 ml) of the

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**Fig. 1. Primary pathways of butadiene monoxide metabolism.**

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maintained at 60°C for 3 min, then increased to 250°C at a rate of 30°C/min. For analysis of DEB, the injection port and detector temperatures were 200 and 280°C, respectively. For analysis of \( \text{meso-} \)- and \( \text{(±)-} \)-DEB, the column temperature was maintained at 80°C for 4 min, then increased to 280°C at a rate of 30°C/min. The final temperature was maintained for 3 min. The injection volume was 3 \( \mu \)l, and injections were made in splitless mode. For detection of DEB isomers, ions \( m/z \) 55 (target ion) and \( m/z \) 85 (qualifier ion) were monitored. Under these conditions, the retention times for \( \text{meso-} \)-DEB, \( \text{(±)-} \)-DEB, and \( \text{cyclohexene oxide} \) were 3.21, 3.30, and 4.86 min, respectively. Following analysis for DEB isomers, sample extracts were derivatized with 25 \( \mu \)l of BSTFA + 10% TMCS for 30 min at 70°C for detection of BDD and butane-1,4-diol (internal standard) as their bis-trimethylsilyl ethers by GC/MS (Kemper et al., 1998). The injection port and detector temperatures were the same as for DEB analysis. The column temperature was maintained at 80°C for 4 min, then increased to 280°C at a rate of 30°C/min. The final temperature was maintained for 3 min. Detection of BDD was accomplished using ions \( m/z \) 129 (target) and \( m/z \) 103 (qualifier), while ions \( m/z \) 116 (target) and \( m/z \) 101 (qualifier) were monitored for butane-1,4-diol (Kemper et al., 1998). Injections (3 \( \mu \)l) were carried out in splitless mode. Under these conditions, BDD and butane-1,4-diol eluted at 4.05 and 5.92 min, respectively. Quantitation of metabolites was achieved by comparing sample target ion peak area ratios (metabolite/internal standard) to standard curves prepared from authentic compounds in reaction buffer and processed exactly as for samples. The limit of detection for both assays was approximately 0.05 nmol/ml.

Quantitation of GSH Conjugates of BMO by LC/MS/MS. Quantitation of GSBMO (mixed isomers) was accomplished using a PerkinElmer Sciex API 365 triple quadrupole LC/MS/MS (PerkinElmer Instruments, Norwalk, CT) coupled to an HPLC. Separation of GSBMO was achieved using a 150-mm \( \times 1\)-mm C\(_{18}\) Hypersil BDS column with 3-\( \mu \)m particle size and 120-A pore size (P.J. Colbert, St. Louis, MO). Chromatographic runs were carried out using 0.05% trifluoroacetic acid in water as solvent A and 95% acetonitrile containing 0.05% trifluoroacetic acid as solvent B. The column was equilibrated at 1% B and then increased linearly to 50% B over 25 min. The percentage of solvent B was then increased to 99% over 10 min, where it was held for 5 min. The solvent composition was then changed back to the initial conditions (1% B) over 5 min. Under these conditions, both regioisomers of GSBMO eluted at 26.4 min. The flow rate was 0.02 ml/min, and the injection volume was 20 \( \mu \)l. For quantitation of GSBMO, the transition of \( m/z \) 378 (M + H) to \( m/z \) 249 (M – glutamate moiety + H) was monitored with a dwell time of 0.2 s. The limit of detection for the assay was 0.31 nmol/ml. No interferences with this reaction were observed in control cell precipitates.

Data Analysis and Statistics. For quantitative comparisons, areas under the curve (AUC) were calculated for time course data. AUCs were estimated by the trapezoidal approximation using the AREA transform of the SigmaPlot software package (SPSS, Chicago, IL). Descriptive statistics were calculated using the SigmaStat software package (SPSS).

Results

Hepatocyte Viability Under Reaction Conditions. Initial viability of hepatocytes isolated by the above procedure was typically 90 to 95%. Preliminary experiments indicated that mouse hepatocytes remained greater than 80% viable for at least 60 min, while rat hepatocytes remained viable for at least 2 h. Furthermore, incubation of mouse and rat hepatocytes for up to 60 min with BMO, \( \text{(±)-} \)-DEB, or BDD at concentrations up to 1 mM did not decrease cell viability compared with control incubations (data not shown). These pilot experiments, which suggested that BMO and its metabolites were not acutely cytotoxic at concentrations well above those used or anticipated in the current study, are consistent with the results previously obtained in freshly isolated rat hepatocytes (Nieusma et al., 1997). Cell viability was assessed by trypan blue exclusion at each time point in the experiments. The data (not shown) verify that hepatocyte viability remained greater than 80% throughout the entire experiment.

Oxidation of BMO to \( \text{meso-} \) and \( \text{(±)-} \)-DEB. The time course of appearance and disappearance of \( \text{meso-} \) and \( \text{(±)-} \)-DEB in the reaction medium following incubation of BMO with mouse and rat hepatocytes is illustrated in Fig. 2. The data from these experiments were used to calculate areas under the concentration versus time curve.
TABLE 1
AUC for BMO metabolites in mouse and rat hepatocytes exposed to BMO.

<table>
<thead>
<tr>
<th>Species</th>
<th>BMO (µM)</th>
<th>AUC0–45 min (nmol · min/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>meso-DEB</td>
<td>(±)-DEB</td>
</tr>
<tr>
<td>Mouse</td>
<td>5</td>
<td>8.7 ± 4.1a</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>43.9 ± 13.1</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>218.2 ± 28.8</td>
</tr>
<tr>
<td>Rat</td>
<td>5</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>13.4 ± 0.5</td>
</tr>
</tbody>
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N.D., not detected.

Values are means ± S.D. for three individual hepatocyte preparations.

(AUC0–45 min), which are presented in Table 1. The AUC provides an index of overall exposure of the cell system to the metabolites of interest. In mouse hepatocytes (Fig. 2A), detectable formation of both meso- and (±)-DEB occurred at all BMO concentrations tested. At the low and middle BMO concentrations (5 and 25 µM), peak levels of the DEB isomers occurred within 10 to 15 min of the addition of substrate and declined thereafter. At 250 µM BMO, the concentration of (±)- and meso-DEB reached an apparent plateau after approximately 30 min. At all substrate concentrations tested, the levels of (±)-DEB were significantly higher than meso-DEB. However, analysis of the AUC data for the DEB isomers and total DEB (Table 1) indicates that the relative amounts of (±)- and meso-DEB varied with initial BMO concentration. As the BMO concentration was increased, the fractional contribution of meso-DEB increased from 13.8 to 54.8% of total DEB.

Flux of BMO through the diepoxide pathway in rat hepatocytes is shown in Fig. 2B. Concentrations of meso- and (±)-DEB measured in rat hepatocytes following exposure to BMO were quite low compared with the results obtained with mouse cells. Indeed, no meso-DEB was detected in rat hepatocytes exposed to 5 µM BMO, whereas this isomer was detectable for at least 20 min at this BMO concentration in mouse hepatocytes. Peak concentrations of DEB in rat hepatocytes generally occurred within 10 min, with the exception of (±)-DEB after the 250 µM BMO exposure, in which case the peak concentration was reached at approximately 20 min. In contrast to mice, DEB had disappeared completely by the end of the experiment, except for (±)-DEB at the highest BMO concentration, which had declined to approximately half of its peak value by the end of the experiment. As with mouse hepatocytes, the fractional contribution of meso-DEB to total DEB increased with increasing BMO concentration (Table 1). However, the effect was less pronounced in rat hepatocytes, with a maximum fractional contribution of approximately 17% at the highest BMO exposure concentration. Overall, total exposure of rat hepatocytes to DEB was markedly lower than in mouse cells exposed to the same concentration of BMO. For example, the AUC0–45 for total DEB in rat cells ranged from 7.8 to 12.9% of the value in mouse hepatocytes over the BMO concentration range examined (Table 1).

Hydrolysis of BMO to BDD. The time course of hydrolysis of BMO to BDD in mouse and rat hepatocytes is shown in Fig. 3, and the AUCs calculated from these data are presented in Table 1. In mouse hepatocytes (Fig. 3A), peak concentrations of BDD occurred at 10, 15, and 40 min following exposure to 5, 25, and 250 µM BMO, respectively. At the low and middle BMO concentrations, the BDD concentration decreased in the latter part of the experiment, while at the highest BMO concentration, BDD reached an apparent plateau after 35 to 40 min. Elimination of BDD appears to occur relatively slowly compared with elimination of meso- and (±)-DEB. A similar pattern of BDD formation was observed in rat hepatocytes exposed to increasing concentrations of BMO (Fig. 3B). As with mouse hepatocytes, the time to peak concentration of BDD in rat hepatocytes varied with the BMO concentration, and elimination of the diol appeared to be quite slow. However, peak concentrations of BDD measured in rat hepatocyte suspensions were approximately 3- to 6-fold higher than in the corresponding mouse experiments. Species differences in flux through the hydrolysis pathway are most clearly seen by comparison...
isoforms of the AUC data for this metabolite (Table 1). The AUC for BDD was approximately 3- to 7-fold greater in rat hepatocytes compared with mouse hepatocytes. For both peak concentration and AUC, the greatest differences were found following the 5 μM BMO exposure.

Conjugation of BMO with GSH. Formation of GSH conjugates of BMO (GSBMO, combined regioisomers) by mouse and rat hepatocytes is illustrated in Fig. 4. In mouse (Fig. 4A), conjugation with GSH occurred very rapidly, with significant levels of GSBMO found even in samples quenched immediately after addition of substrate. Peak levels were reached within 5 min and appeared to decline slightly with time thereafter. However, because of the high variability of these measurements, the apparent decline may be an artifact. In rat hepatocytes (Fig. 4B), GSBMO formation appears to proceed more slowly, with peak levels occurring after 10 to 20 min, depending on the BMO concentration. Similar peak concentrations of conjugates were observed in rats compared with mice, but the apparent decline in conjugate concentration with time seen in mouse hepatocytes was not evident in rats. Comparison of AUC values for GSBMO formation (Table 1) suggests that similar amounts of BMO were metabolized via this pathway in both species. For both mice and rats, there was an approximately linear increase in AUC for GSBMO with increasing initial BMO concentration.

Discussion

Isolated mouse and rat hepatocytes catalyzed oxidation of BMO to DEB. Once formed, DEB may be eliminated via hydrolysis to epoxybutanediol and erythritol and by GSH conjugation, catalyzed by GSTs. The former reaction was observed in rat and human liver microsomes (Krause and Elfarra, 1997), while the latter reaction was demonstrated in cytosolic preparations from mouse and rat tissues (Boogaard et al., 1996; Nieuwena et al., 1998). While both meso- and (±)-DEB were detected in the mouse and rat hepatocytes, (±)-DEB was preferentially detected over (−)-DEB at the three BMO concentrations used, although (±)-DEB detection increased at high BMO concentrations (Table 1; Fig. 2). These results are consistent with our previous results showing a similar concentration dependence in the stereoselectivity of DEB detected from BMO in mouse and rat liver microsomes (Krause and Elfarra, 1997). In that study, (−)-DEB was found to be hydrolyzed more efficiently by rat liver microsomal EH compared with (±)-DEB (Krause and Elfarra, 1997), whereas mouse liver microsomes displayed little hydrolytic activity toward both DEB isomers. Collectively, these findings are consistent with the rapid and relatively complete elimination of DEB from rat hepatocytes compared with mouse hepatocytes (Fig. 2).

DEB can also be conjugated with GSH, both enzymatically, and to a lesser extent by direct reaction (Boogaard et al., 1996; Nieuwena et al., 1998). In liver cytosol from rats and mice, conjugation is apparently followed by rapid hydration of the remaining epoxide moiety, leading to formation of trihydroxybutylglutathione conjugates (Boogaard et al., 1996). Because species comparisons demonstrated little difference in DEB-GSH conjugation kinetics between cytosolic preparations from mice and rats (Boogaard et al., 1996), formation of GSH conjugates probably contributes little to species differences in DEB levels detected in hepatocytes incubated with BMO. Rather, the major determinants of mouse and rat differences in DEB exposure in BMO-treated hepatocytes are likely to be rates of BMO oxidation and DEB hydrolysis. Overall, the hepatocyte data suggest that at equivalent BMO doses, DEB exposure in rats in vivo will be significantly lower than in mice.

Mouse and rat hepatocytes readily hydrolyzed BMO to BDD (Fig. 3). Small amounts of BDD were detected in the urine of animals exposed to BD (Nauhaus et al., 1996; Anttinen-Klemetti et al., 1999) and BMO (Krause et al., 1997). Enzymatic hydrolysis of BMO has been demonstrated directly (Krause et al., 1997) and indirectly (Kreuzer et al., 1991; Csanády et al., 1992) in liver microsomal and cytosolic preparations from mice and rats. BMO microsomal hydrolysis is approximately 3-fold more efficient in rat liver microsomes compared with mouse liver microsomes (Krause et al., 1997). No cystolic EH activity toward BMO was observed in rat liver, whereas the mouse microsomal and cystolic BMO EH activities were comparable under saturating substrate concentrations (Krause et al., 1997). These results suggest the cystolic form of EH may contribute significantly to BMO hydrolysis in mouse hepatocytes. Relatively slow elimination of BDD from both rat and mouse hepatocytes was observed in the current study (Fig. 3). In vitro, BDD is metabolized by cystolic alcohol dehydrogenases (Kemper and Elfarra, 1996) and by cytochrome P450s (Sabourin et al., 1992). Dehydrogenation of BDD, presumably to 2-hydroxy-3-butenal, is a low-affinity, high-capacity pathway in mouse and rat liver cytosol (Kemper and Elfarra, 1996); the efficiency of this in vitro reaction was similar in the two species (V_{max}/K_m = 0.53–0.66). NADPH-dependent oxidation of BDD by mouse and rat liver microsomes led to formation of hydroxymethylvinyl ketone (Kemper et al., 1997). This compound is thought to be the precursor to 1,2-dihydroxy-4-(N-acetylcysteiny)butane, the pri-
mary urinary metabolite detected in monkeys and humans exposed to BD (Sabourin et al., 1992; Bechtold et al., 1994).

Another major pathway for BMO metabolism is conjugation with GSH, catalyzed by cytosolic GSTs, resulting in the formation of regioisomers of GSBMO. Previous studies have demonstrated formation of both regioisomers of GSBMO both in vitro (Sharer et al., 1991, 1992; Csánády et al., 1992) and in vivo (Sabourin et al., 1992; Sharer and Elfarra, 1992; Bechtold et al., 1994; Elfarra et al., 1995), but the HPLC detection methods used in these studies were not sensitive enough to quantitate the GSH conjugates of BMO in isolated hepatocytes. Both regioisomers of GSBMO were detected by our LC/MS methodology, although we were unable to resolve these regioisomers to permit their individual quantitation. The results indicate that similar amounts of BMO were metabolized by GSH conjugation in mouse and rat hepatocytes. This is consistent with previous studies from our laboratory, which showed that mice and rats dosed with BMO (20 mg/kg, i.p.) excreted similar amounts of combined S-(2-hydroxy-3-buten-1-yl)-N-acetyl-L-cysteine and S-(1-hydroxy-3-buten-2-yl)-N-acetyl-L-cysteine in the urine within 24 h (Elfarra et al., 1995).

Likewise, in vitro kinetic experiments using mouse and rat liver cytosol revealed similar maximal formation rates and $V_{\text{max}}/K_m$ ratios from these species (Csánády et al., 1992; Sharer et al., 1992). Thus, conjugation of BMO with GSH does not appear to be a major determinant of differences in BMO toxicity in mice and rats.

In the current studies, the AUCs for metabolite time courses were used as an index of overall exposure of the cell to each metabolite and as a measure of flux of BMO through each of its major metabolic pathways. Since recovery experiments indicated that DEB and BDD (10–30 μM) were rapidly and uniformly distributed throughout the hepatocyte suspension, these metabolites were measured in the reaction medium instead of the intact or homogenized suspension. Because the distribution of metabolites at lower concentrations within the system was not determined, it is possible that at early time points in the experiments, intracellular metabolites may not have fully equilibrated with the extracellular medium, resulting in underestimation of AUCs. Nevertheless, since concentrations of metabolites measured in the medium primarily represent concentrations in the whole suspensions averaged over the course of the experiment, and since any underestimation at early time points would be similar in both species tested, the results of our comparative analysis are still valid. While separate experiments were carried out to quantitate GSBMO formation, the metabolic system could easily be scaled up such that all three metabolites could be measured in the same cell suspension.

The observed species differences in the AUCs in our studies reflect species differences in both metabolism and toxicity of mice and rats exposed to BD (Owen et al., 1987; Melnick et al., 1990; Himmelstein et al., 1995; Himmelstein et al., 1997; Thorton-Manning et al., 1997). Activation-detoxication ratios for BMO may be calculated by dividing the AUC for total DEB by the summed AUC values for GSBMO and BDD at each concentration (Table 1). In mouse hepatocytes, there is an inverse relationship between exposure concentration and activation-detoxication ratio. At the lowest concentration, bioactivation of BMO exceeds detoxication capacity by approximately 2-fold, whereas at the highest concentration, activation is only about 31% of total detoxication. In rat hepatocytes, the activation-detoxication ratio is relatively independent of the initial BMO concentration, with flux through the oxidative pathway at approximately 2 to 5% of total detoxication. Thus, depending on the exposure concentration, the activation-detoxication ratio for BMO in mouse hepatocytes was between 15- and 40-fold higher than in rat hepatocytes. These results, which are consistent with the reported 40-fold higher blood levels of DEB in mice exposed to BD (62 ppm) in comparison with the rat blood levels (Himmelstein et al., 1997; Thorton-Manning et al., 1997), suggest that even in the absence of rate-limiting formation of BMO from BD, mice would be more sensitive to the carcinogenic effects of BMO compared with rats, particularly at lower BMO concentrations. Because mouse liver microsomes are also more effective in BD oxidation to BMO in comparison with rat liver microsomes (Csánády et al., 1992; Duescher and Elfarra, 1994), freshly isolated hepatocytes would probably exhibit even greater species differences in bioactivation of BMO to DEB than that observed with BMO in the present study.

Studies using hepatocytes complement subcellular metabolism studies by providing a significant increase in biological complexity while still allowing the high degree of control characteristic of simpler in vitro systems. In the present study, rodent hepatocytes catalyzed formation and elimination of all three of the major biotransformations of BMO characterized in subcellular kinetic studies: oxidation to meso- and (±)-DEB, hydrolysis to BDD, and conjugation with GSH. These results, which reflect in vivo metabolic handling of BMO (Himmelstein et al., 1995, 1997; Thorton-Manning et al., 1997), are more consistent with mouse and rat in vivo toxicity data than the in vitro kinetic studies in rodent subcellular fractions (Kreuzer et al., 1991; Sharer et al., 1992; Seaton et al., 1995; Boogaard et al., 1996; Krause and Elfarra, 1997; Krause et al., 1997). Hence, intact cells are useful for bridging the gap between subcellular and whole animal systems and may provide a more realistic picture of overall in vivo metabolism. Prolonged inhalation exposure of mice to BD (625 and 1250 ppm) has been associated with blood BMO concentrations of 4 to 8 μM, respectively (Himmelstein et al., 1995, 1997), which are comparable with the low BMO concentration used in the current study.

The isolated hepatocyte approach combined with sensitive analytical methods allows a great deal of information to be extracted from a few experiments. Although the current studies focused on primary metabolites of BMO, this approach could be used to examine production of secondary and tertiary metabolites, such as epoxybutanediol and hydroxymethylvinyl ketone, and to study formation of covalent adducts with nucleophilic targets in cellular macromolecules. These experiments, however, require the development of highly sensitive analytical methods appropriate for use in the isolated cell model. The consistency between our results and the carcinogenic susceptibility of mice and rats to BD suggests that isolated hepatocytes may be a useful model for predicting human susceptibility to BD and its metabolites. The increasing commercial availability of cryopreserved human hepatocytes should allow further development of this model and its application to human risk assessment. Clearly, further studies are needed to realize the full potential of isolated hepatocyte models for investigation of the role of metabolism in species differences in metabolism and toxicity of BD and related compounds.

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