DETECTION OF CARBOXYLIC ACIDS AND INHIBITION OF HIPPURIC ACID FORMATION IN RATS TREATED WITH 3-BUTENE-1,2-DIOL, A MAJOR METABOLITE OF 1,3-BUTADIENE

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(Received January 27, 2003; accepted April 25, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

Epidemiological studies have indicated that 1,3-butadiene exposure is associated with an increased risk of leukemia. In human liver microsomes, 1,3-butadiene is rapidly oxidized to butadiene monoxide, which can then be hydrolyzed to 3-butene-1,2-diol (BDD). In this study, BDD and several potential metabolites were characterized in the urine of male B6C3F1 mice and Sprague-Dawley rats after BDD administration (i.p.). Rats given 1420 or 2840 \(\mu\)mol kg\(^{-1}\) BDD excreted significantly greater amounts of BDD relative to rats administered 710 \(\mu\)mol kg\(^{-1}\) BDD. Rats administered 1420 or 2840 \(\mu\)mol kg\(^{-1}\) BDD excreted significantly greater amounts of BDD per kilogram of body weight than mice given an equivalent dose. Trace amounts of 1-hydroxy-2-butanone and the carboxylic acid metabolites, crotonic acid, propionic acid, and 2-ketobutyric acid, were detected in mouse and rat urine after BDD administration. Because of the identification of the carboxylic acid metabolites and because of the known ability of carboxylic acids to conjugate coenzyme A, which is critical for hippuric acid formation, the effect of BDD treatment on hippuric acid concentrations was investigated. Rats given 1420 or 2272 \(\mu\)mol kg\(^{-1}\) BDD had significantly elevated ratios of benzoic acid to hippuric acid in the urine after treatment compared with control urine. However, this effect was not observed in mice administered 1420 or 2840 \(\mu\)mol kg\(^{-1}\) BDD. Collectively, the results demonstrate species differences in the urinary excretion of BDD and show that BDD administration in rats inhibits hippuric acid formation. The detection of 1-hydroxy-2-butanone and the carboxylic acids also provides insight regarding pathways of BDD metabolism in vivo.

1,3-Butadiene (BD\(^3\)) is an industrial chemical used extensively in the manufacturing of a variety of synthetic rubbers and plastics. It is estimated that 65,000 workers in the United States are exposed annually to BD (Federal Register, 1990). BD has also been detected in cigarette smoke, automobile exhaust, and urban air (Brunneman et al., 1990). Epidemiological studies have shown an increased incidence of hematopoietic cancer among workers exposed to BD (Santos-Burgoa et al., 1992; Landrigan, 1993). In 2000, the U.S. Department of Health and Human Services-National Toxicology Program classified BD as a “known human carcinogen” (U.S. Department of Health and Human Services, section 111-14). Long-term inhalation studies in mice and rats resulted in multisite tumors in both species, although mice exhibited a higher susceptibility to BD (Owen et al., 1987; Melnick et al., 1990). This research was supported by National Institutes of Health Grant ES06841. C.L.S. was supported by a chemistry-biology interface training grant from the National Institutes of Health (GM08505-08).

Abbreviations used are: BD, 1,3-butadiene; BMO, butadiene monoxide; BDD, 3-butene-1,2-diol; HBO, 1-hydroxy-2-butanone; ADH, alcohol dehydrogenase; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane; HMVK, hydroxymethylvinylketone.

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BD initially is enzymatically converted to butadiene monoxide (BMO) via cytochromes P450 (Duescher and Elfarra, 1994; Himmelstein et al., 1994) and myeloperoxidase (Duescher and Elfarra, 1992). BMO can be either hydrolyzed to 3-butene-1,2-diol (BDD) by cytosolic or microsomal epoxide hydrolase (Kemper and Elfarra, 1996; Krause et al., 1997), a major metabolic pathway in humans (Bechtold et al., 1994), or oxidized by cytochromes P450 to form diepoxynbutane (Csany et al., 1992; Krause and Elfarra, 1997). BD toxicity is currently thought to be associated with the epoxide metabolites BMO and diepoxynbutane (Bechtold et al., 1994; Xi et al., 1997; Saranko et al., 1998).

Although hydrolysis of BMO to BDD is considered a detoxification pathway of BMO (Himmelstein et al., 1994), BDD’s role in BD toxicity is unclear. This is largely due to limited research on the in vivo metabolism (Kemper et al., 1998) and toxicity of BDD. Previous studies in our laboratory showed that mice treated with BDD (10–250 mg kg\(^{-1}\)) excreted only 2 to 5% of the dose as BDD (Kemper et al., 1998). However, the fate of the remainder of the BDD dose was unclear. Earlier studies in our laboratory (Kemper and Elfarra, 1996) illustrated an in vitro metabolism of BDD. That previous study showed the formation of 1-hydroxy-2-butanone (HBO) from BDD and suggested the involvement of multiple steps involving alcohol dehydrogenase (ADH; Kemper and Elfarra, 1996). However, it was not known whether HBO is an in vivo metabolite of BDD in mice or rats.

Production of carboxylic acids from BDD in mice and rats is hypothesized to occur via the metabolic pathway suggested by Kem-
Fig. 1. Possible pathways of 3-butene-1,2-diol (BDD) metabolism by alcohol dehydrogenase (pathway 1), or sulfotransferases or glucuronosyl-transferases (pathway 2).

ALDH, aldehyde dehydrogenase. The potential BDD metabolites investigated in this study were 1-hydroxy-2-butanone, 2-ketobutyric acid, propionic acid, vinlyacetic acid, and crotonic acid.
per and Elfarra (1996) (Fig. 1, pathway 1), and from the formation of unstable glucuronide/sulfate conjugates (Fig. 1, pathway 2). In pathway 1, ADH-mediated oxidation of BDD can lead to the formation of 2-ketobutanal which can be reduced by ADH in the presence of NADH to form HBO (Kemper and Elfarra, 1996). The 2-ketobutanal intermediate may also be oxidized by aldehyde dehydrogenase to form 2-ketobutyric acid. Metabolic studies of 2-ketobutyrlic acid in rat hepatocytes and homogenate indicate that 2-ketobutyric acid will readily decarboxylate, resulting in the formation of propionic acid (Steele et al., 1984; Lapointe and Olson, 1985).

The allylic hydroxy group of BDD may undergo glucuronidation or sulfate conjugation (Fig. 1, pathway 2). Solvolysis of these conjugates may then occur because of the expected formation of a stable allylic carbonium ion in the transition state of the reaction (Michejda and Koepeke, 1994). However, the allylic carbonium ion may also undergo a 1,2-hydride shift to form 3-butenal, which could be oxidized by aldehyde dehydrogenase to form vinylacetic acid. The proximity of the internal methylene moiety of 3-butenal to the vinyl and aldehyde moieties makes these internal hydrogens very acidic. As a result, 3-butenal could readily tautomize to form crotonaldehyde (Elfarra et al., 1991; Duesscher and Elfarra, 1993). Crotonaldehyde could then be oxidized to form crotonic acid via aldehyde dehydrogenase. Therefore, crotonic acid, vinylacetic acid, 2-ketobutyric acid, and propionic acid represent possible carboxylic acid metabolites of BDD. These acids and HBO may be present in conjugated or free forms in the urine.

The goal of this study was to investigate species differences in the urinary excretion of BDD and its metabolites in male B6C3F1 mice and Sprague-Dawley rats. Specifically, the metabolites investigated included propionic acid, crotonic acid, vinylacetic acid, and HBO, and their respective glucuronide/sulfate conjugates. Male B6C3F1 mice and Sprague-Dawley rats were used based on their known differences in the metabolism of BDD (Richardson et al., 1999) and BMO (Sharer et al., 1992; Krause and Elfarra, 1997). The use of multiple species can better indicate the relative prevalence of specific BDD metabolic pathways.

Materials and Methods

Chemicals and Reagents. Racemic BDD was obtained from Acros Chemicals (Pittsburgh, PA). Acrylic acid, crotonic acid, 2-ketobutyric acid, isobutyric acid, propionic acid, vinylacetic acid, HBO, hippuric acid, benzoic acid, and methylated by diazomethane. Each acid was then later detected in urine using single ion mode. The ions used for detection of methyl propionate (methylated propionic acid) were at m/z 29 ([CH 3 CH 2 COO] - ), 57 ([M-CH 3 ] - ), and 88 ([M] + ); for methyl crotonate, 41 ([CHCO] - ) and [CH 2 CHCHO] - , 69 ([M-CH 3 ] - ), and 100 ([M] + ); for methyl 2-ketobutyrate, 29 ([CH 3 CH 2 COO] - ), 57 ([M-(O)CH 2 COO] - ), and 116 ([M] + ); for methyl isobutyrate, 41 ([CHCO] - ), 43 ([CH 2 CH 2 CHCHO] - ), 71 ([M-OCH 3 ] - ), and 87 ([M] + ); and for methyl 3-butenoate, 41 ([CH 3 CH 2 CHCHO] - ), 59 ([O=CHCHO] - ), and 71 ([M-CH 3 CH] - ). The concentration of each acid in urine was quantitated by calculating the peak area ratio of each acid to isobutyric acid. Values were corrected for any acids found in the urine prior to treatment. Standard curves (r > 0.99) of urine spiked with each acid along with isobutyric acid were prepared as indicated above. The limits of detection for vinylacetic acid and crotonic acid were 1 nmol ml -1 for 2-ketobutyric acid, 5 nmol ml -1 for propionic acid, 25 nmol ml -1.

For BDD and HBO analysis, the initial oven temperature was 70°C for 5 min. The temperature was then raised at a rate of 30°C/min to 250°C and held for 5 min. The retention times of BDD, 2-butenoate, and HBO were 7.84, 8.63, and 6.80 min, respectively. BDD and HBO were originally identified based upon retention times and MS spectra of authentic standards silylated via BSTFA in ethyl acetate or methyl chloride, respectively. BDD, 2-buten-1,4-diol, and HBO were detected using single ion mode. Trimethylsilyl TMS derivatives of BDD were detected using ions at m/z 103 ([TMSOCH 3 ] - ), 129 ([M-CH 3 OCH 3 ] - ), and 217 ([M-CH 3 ] - ); for 2-butenoate, 129 ([M-CH 3 OCH 3 ] - ), 142 ([M-CH 3 CH] - ), and 217

BDD and HBO Analysis. BDD was extracted from urine and derivatized with BSTFA with 10% TMCS as described previously (Kemper et al., 1998). Methods for HBO extraction and derivatization were based on the methods previously described by Kemper et al. (1998) with slight modifications. Briefly, an aliquot of urine (350 μl) and 100 μl of 10 μg/ml 2-butene-1,4-diol as internal standard were placed on the solid-phase Extrelut packing material inside a glass column (1 g). After 5 min, two 3-ml aliquots of the urine were added to each column. The combined ethanol chloride extracts were concentrated to 100 to 200 μl under N2 and then derivatized with BSTFA with 10% TMCS. For HBO glucuronide and sulfate conjugates, β-glucuronidase (5000 units) and sulfatase (300 units) were added to the urine sample (350 μl). Aliquots of the same urine with no added enzyme were run concurrently to determine the increase in concentration of each carboxylic acid after β-glucuronidase/sulfatase treatment relative to urine without enzymatic treatment. The solutions were incubated for 20 h at 37°C and then extracted and derivatized as described above.

Carboxylic Acid Analysis. A method was developed to analyze for propionic acid, crotonic acid, vinylacetic acid, and 2-ketobutyric acid by GC/MS. Urine (900 μl) was combined with 100 μl of 200 μM isobutyric acid as internal standard. Urine was then acidified to pH 1.8 ± 0.05 with 1 mM HCl, and 650 to 700 mg of NaCl was added. The solution was then extracted with methylene chloride (three times, 2 ml). The extracts were concentrated to 2 ml under N2 and derivatized with ethereal diazomethane. The solutions were then further concentrated to 200 μl under N2 before analysis by GC/MS as described below. Some samples were treated with β-glucuronidase (5000 units) and sulfatase (300 units) and incubated at 37°C for 20 h. Control samples without enzyme were also run to determine whether the acid concentration changed during the assay. The samples were extracted with methylene chloride, derivatized, and analyzed for free acids as described above.

GC/MS Conditions. Analyses of the carboxylic acid, BDD, and HBO were performed on a Hewlett Packard series 6890 gas chromatograph with a mass selective detector. The gas chromatograph was fitted with a DB-1 column (J&W Scientific, Folsom, CA). The injection port temperature was 250°C with a pressure of 1.55 psi. Injections (3 μl) were made in splitless mode at a column head pressure of 1.6 psi. For the carboxylic acid analysis, the initial oven temperature was set to 36°C. The temperature then increased at a rate of 70°C/min to 70°C, then 10°C/min to 90°C, 40°C/min to 130°C, and finally increased 70°C/min to 220°C where it was held for 1 min. Under these conditions, the retention times for propionic acid, isobutyric acid, vinylacetic acid, crotonic acid, and 2-ketobutyric acid were 2.75, 3.66, 3.95, 4.57, and 5.40 min, respectively (Fig. 2).

Metabolites were initially identified based upon retention times and MS fragments from authentic standards spiked into urine. Each acid was then later detected in urine using single ion mode. The ions used for detection of methyl propionate (methylated propionic acid) were at m/z 29 ([CH 3 CH 2 COO] - ), 57 ([M-OCH 3 ] - ), and 88 ([M] + ); for methyl crotonate, 41 ([CHCO] - ) and [CH 2 CHCHO] - , 69 ([M-CH 3 ] - ), and 100 ([M] + ); for methyl 2-ketobutyrate, 29 ([CH 3 CH 2 COO] - ), 57 ([M-(O)CH 2 COO] - ), and 116 ([M] + ); for methyl isobutyrate, 41 ([CHCO] - ), 43 ([CH 2 CH 2 CHCHO] - ), 71 ([M-OCH 3 ] - ), and 87 ([M] + ); and for methyl 3-butenoate, 41 ([CH 3 CH 2 CHCHO] - ), 59 ([O=CHCHO] - ), and 71 ([M-CH 3 CH] - ). The concentration of each acid in urine was quantitated by calculating the peak area ratio of each acid to isobutyric acid. Values were corrected for any acids found in the urine prior to treatment. Standard curves (r > 0.99) of urine spiked with each acid along with isobutyric acid were prepared as indicated above. The limits of detection for vinylacetic acid and crotonic acid were 1 nmol ml -1 for 2-ketobutyric acid, 5 nmol ml -1 for propionic acid, 25 nmol ml -1.

For BDD and HBO analysis, the initial oven temperature was 70°C for 5 min. The temperature was then raised at a rate of 30°C/min to 250°C and held for 5 min. The retention times of BDD, 2-butene-1,4-diol, and HBO were 7.84, 8.63, and 6.80 min, respectively. BDD and HBO were originally identified based upon retention times and MS spectra of authentic standards silylated via BSTFA in ethyl acetate or methyl chloride, respectively. BDD, 2-buten-1,4-diol, and HBO were detected using single ion mode. Trimethylsilyl TMS derivatives of BDD were detected using ions at m/z 103 ([TMSOCH 3 ] - ), 129 ([M-CH 3 OCH 3 ] - ), and 217 ([M-CH 3 ] - ); for 2-butene-1,4-diol, 129 ([M-CH 3 OCH 3 ] - ), 142 ([M-CH 3 CH] - ), and 217
Peak I, propionic acid; peak II, isobutyric acid (internal standard); peak III, crotonic acid; peak IV, 2-ketobutyric acid. Unlabeled peaks represent unknown urinary components that were not consistently present in urine or unaffected by BDD treatment.

((M-CH₃)⁺); and for HBO, 105 (M-CH₃CH₂ and 2(CH₃)⁺). Concentrations of HBO and BDD were determined by calculating a ratio of HBO or BDD to 2-butene-1,4-diol in urine before and after BDD treatment. Samples were corrected for background concentrations found in the control urine. The resulting ratio was compared to a ratio obtained from standard curves (r ≥ 0.99) of urine spiked with HBO or BDD along with 2-butene-1,4-diol prepared as indicated above. The limits of detection for BDD and HBO were 0.1 and 10 μmol ml⁻¹, respectively.

HPLC Analysis of Benzoic Acid and Hippuric Acid. Urine samples (50 μL) were added to deionized water (950 μL). The diluted samples were then deproteinized with 30 μL of iced concentrated HCl. Samples were centrifuged at 3000 rpm, and the supernatant was analyzed on a gradient-controlled HPLC system (Gilson Medical Electronics, Middleton, WI) equipped with a Brownlee Newguard guard column (Rainin Instruments, Woburn, MA) and a Beckman system Gold 166 variable wavelength detector (Beckman Coulter Inc., Fullerton, CA), using a Beckman Ultrasphere 5-μm ODS reverse-phase analytical column (4.6 mm × 25 cm) with UV detection at 229 nm. Samples (20 μL) were separated using 1% acetonitrile adjusted to pH 2.5 with trifluoroacetic acid as a mobile phase for pump A and 75% acetonitrile adjusted to pH 2.5 with trifluoroacetic acid as a mobile phase for pump B. The flow rate was 1 ml min⁻¹. Initially 10% B was maintained for 5 min. The gradient then increased from 10% B to 60% B from 5 to 8 min, where it was then held for 3 min. The percentage of mobile phase B was then decreased from 60% to 10% B from 11 to 14 min and held for 6 min. Hippuric acid and benzoic acid eluted at 10.5 and 12.5 min, respectively. Concentrations of hippuric acid and benzoic acid were determined by comparing the peak area of each acid with standard curves prepared of known concentrations. Due to the presence of hippuric acid and benzoic acid in all urine samples, the standard curves were prepared in water to quantitate the total levels of hippuric acid and benzoic acid in each sample. The area response for both acids was linear from 2 to 2000 nmol ml⁻¹ (r > 0.99). The limits of detection for both benzoic acid and hippuric acid were 2 nmol ml⁻¹.

Statistical Analyses. All results were analyzed using Sigmastat software (SPSS Inc., Chicago, IL). A paired t test was used to determine significance between two groups. To determine significant difference between multiple groups of data, a one-way analysis of variance test was used.

Results

BDD Detection in Urine. BDD was detected in the urine of mice and rats after BDD administration. Of the total BDD amounts excreted in the urine 0 to 24 h after treatment, mice and rats excreted greater than 75 and 90%, respectively, in the first 8 h (data not shown). There were no significant differences between the amounts of free and total BDD excreted in mouse or rat urine within each treatment (Fig. 3). Rats administered a dose of 1420 μmol kg⁻¹ BDD excreted significantly more BDD compared with rats given 710 μmol kg⁻¹ BDD, whether the data were expressed as μmol kg⁻¹ (Fig. 3) or as the percentage of the dose (p < 0.05; data not shown). However, rats treated with 2272 or 2840 μmol kg⁻¹ BDD excreted similar amounts of BDD relative to the 1420 μmol kg⁻¹ dose (Fig. 3). When the latter data are expressed as the percentage of the dose, rats given the 1420 μmol kg⁻¹ dose excreted a significantly higher percentage of the dose as total BDD (10.8%) compared with rats treated with 2272 or 2840 μmol kg⁻¹ BDD (4.9 and 4.7%, respectively; p < 0.05). The lack of dose-dependent excretion of the total amounts of BDD between the four separate doses administered to rats may possibly be due to saturation of BDD elimination at the two highest doses.

Mice treated with 1420 or 2840 μmol kg⁻¹ BDD excreted similar amounts of BDD whether the data were expressed as μmol kg⁻¹ (Fig. 3) or as the percentage dose recovered as total BDD (3.4 and 1.7%, respectively). Compared to mice treated with 1420 and 2840 μmol kg⁻¹ BDD, rats given an equivalent dose excreted a significantly higher total amount (μmol kg⁻¹) of BDD (p < 0.05; Fig. 3). However, the amount of free BDD excreted by rats given 2840 μmol kg⁻¹ BDD was not significantly different from the amount of free BDD excreted by mice given an equivalent dose.

Detection of HBO and Carboxylic Acid Metabolites of BDD. In both mice and rats treated with BDD, three carboxylic acid urinary metabolites of BDD were identified based upon comparisons of
GC/MS retention time and mass spectra with known standards (propionic acid, crotonic acid, and 2-ketobutyric acid; Fig. 2). Vinylic acid was not detected in any sample. Propionic acid was present in urine collected before treatment. However, there was a 2- and 3-fold increase in the amounts of propionic acid excreted after mice were given 1420 or 2840 μmol kg⁻¹ BDD, respectively. The amount of propionic acid present in rat urine also increased 2- and 6-fold after administration of 1420 and 2840 μmol kg⁻¹ BDD, respectively (Fig. 2). Crotonic acid and 2-ketobutyric acid were only detected in urine samples after treatment with BDD and were not present in the control urine (Fig. 2). There were no significant differences in the amounts of propionic acid, crotonic acid, or 2-ketobutyric acid when comparing the levels of the three acids at the same dose of BDD within the same species (Table 1). There were no significant differences in the levels of each individual acid between doses within each species or between species given equivalent doses. Furthermore, there were no significant differences in the combined amounts of propionic acid, crotonic acid, 2-ketobutyric acid, and HBO between the different BDD doses within each species and between species given equivalent doses. The combined concentration for all acids for each treatment and species accounted for <1.0% of the dose. The finding that the amounts of carboxylic acids detected before and after enzymatic hydrolysis were similar suggests that only trace levels of glucuronide or sulfate conjugates of the carboxylic acids may have been formed.

Both rats and mice excreted low levels of HBO as a result of BDD treatment (doses of 1420 or 2840 μmol kg⁻¹, Table 1). HBO was not detected in the urine of mice given 1420 μmol kg⁻¹ BDD (Table 1).

**Ratio of Benzoic Acid to Hippuric Acid.** Because of the identification of the carboxylic acid metabolites and their known ability to form coenzyme A (CoA) esters (Lapointe and Olson, 1985; Brass et al., 1986; Rognstad, 1991) and because CoA is necessary for conjugation of benzoic acid with glycine to form hippuric acid (Gatley and Sherratt, 1977), the effect of BDD treatment on benzoic acid and hippuric acid levels was investigated. Rats treated with 1420 or 2272 μmol kg⁻¹ BDD excreted a significantly higher ratio of benzoic acid to hippuric acid in the urine 0 to 24 h after treatment relative to control urine (Fig. 4). There was no difference between the ratios of benzoic acid to hippuric acid in the urine of rats treated with 710 μmol kg⁻¹ relative to control urine. Also, there were no significant differences in the ratios of benzoic acid to hippuric acid in the urine before treatment in rats dosed with 710, 1420, or 2272 μmol kg⁻¹ BDD. However, rats dosed with 1420 and 2272 μmol kg⁻¹ BDD excreted a significantly higher ratio of benzoic acid to hippuric acid in the urine 0 to 24 h after treatment when comparing the urine 0 to 24 h after dosage in rats treated with 710 μmol kg⁻¹ (p < 0.05). There was no significant difference in the ratio of benzoic acid to hippuric acid in the urine 0 to 24 h after treatment of rats with 1420 or 2272 μmol kg⁻¹ BDD. In mice administered 1420 or 2840 μmol kg⁻¹ BDD, there was no significant difference between the ratio of benzoic acid to hippuric acid before and after treatment.

**Discussion**

The results in this paper demonstrate novel pathways of BDD metabolism leading to the formation of propionic acid, crotonic acid, and 2-ketobutyric acid in mice and rats. Although the levels of propionic acid, crotonic acid, 2-ketobutyric acid, and HBO are low, they do provide evidence for the pathways proposed in this paper (Fig. 1). In the case of pathway 2 in Fig. 1, production of crotonic acid as a result of BDD exposure suggests the formation of crotonaldehyde. Crotonaldehyde, a Michael acceptor, is a known rodent toxin (Chung et al., 1986; Eder and Eder, 2000), and our laboratory has detected crotonaldehyde in mouse liver microsomes incubated with BD (Elfarra et al., 1991; Sharer et al., 1992). Crotonic acid formation may be low because the oxidation of crotonaldehyde to crotonic acid is competing with other metabolic pathways, such as glutathione conjugation (Gray and Barnsley, 1971) or nonspecific addition of crotonaldehyde to cellular nucleophiles. The identification of crotonic acid is significant because crotonaldehyde production from BDD could indicate that metabolism of BDD represents an additional bioactivation pathway for BD.

2-Ketobutyric acid, propionic acid, and HBO are believed to be products of the same metabolic pathway (Fig. 1, pathway 1). Their low levels indicate that there may be competition for each step in the pathway. Our laboratory showed that the primary and secondary hydroxyl group of BDD can be selectively oxidized by ADH and P450s, respectively (Kemper and Elfarra, 1996; Krause et al., 2001). The oxidation of the secondary hydroxyl moiety of BDD results in the formation of hydroxymethylvinylketone, a Michael acceptor, and a very reactive compound. Oxidation of BDD to hydroxymethylvinylketone may be competing with oxidation of the primary hydroxyl of BDD that results in the formation of propionic acid, crotonic acid, and HBO.

The formation of propionic acid from BDD may also elucidate the mechanism of formation of CO₂, a BD-derived metabolite. Richardson et al. (1999) administrated ¹⁴C-labeled BD to mice and rats and noted a significant amount of radioactivity exhaled as ¹⁴C-labeled CO₂ with rats exhaling roughly 3 times the concentration (μCi kg⁻¹) of ¹⁴C-labeled CO₂ relative to mice receiving an equivalent dose. However, the source of the CO₂ was not clear. The decarboxylation of 2-ketobutyric acid to propionic acid may likely be contributing to the increase in exhaled CO₂ observed in rats. This notion is further supported by the higher rates of BMO hydrolysis observed in rats as
Materials and Methods

Urine samples were analyzed for benzoic acid and hippuric acid as outlined under Materials and Methods. Values are expressed as means ± S.D. Doses of 710, 1420, 2272, and 2840 μmol kg⁻¹ correspond to 62.5, 125, 200, and 250 mg kg⁻¹, respectively. A, significant difference (p < 0.05) between the ratios of benzoic acid to hippuric acid in the urine prior to treatment and urine 0 to 24 h after exposure within a treatment group. B, significant difference (p < 0.05) in the ratio of benzoic acid to hippuric acid in rat urine 0 to 24 h after dosing, relative to the urine 0 to 24 h after treatment in rats dosed with 710 μmol kg⁻¹ BDD.

compared with mice (Krause et al., 1997). The higher rates of BDD formation in rats could lead to a greater production of 2-ketobutyric acid and thus be contributing to the greater formation of CO₂ in rats relative to mice.

Overall, the levels of the carboxylic acids recovered in the urine were low, but this may be due to further metabolism of the carboxylic acids. Multiple studies have indicated that conjugation with CoA is a major pathway for the metabolism of carboxylic acids such as propionic acid and 2-ketobutyric acid (Lapointe and Olson, 1985; Brass et al., 1986; Rognstad, 1991). CoA is a critical intermediate in the conjugation of benzoic acid, a common component of rodent feed and a breakdown product of aromatic amino acids, with glycine to produce hippuric acid (Gatley and Sherratt, 1977). However, unlike benzoic acid, these small carboxylic acids do not readily form glycine conjugates because they do not have the appropriate functional groups (arylacetic, aryloxyacetic, aromatic, and heteroaromatic carboxylic acids) for glycine conjugation (Knights, 1998). Also, short-chain carboxylic acid CoA esters have been shown to inhibit the enzymes and transporters necessary for the conjugation of carboxylic acids with CoA (Sherratt, 1985; Matsuishi et al., 1991). Therefore, hippuric acid formation may be inhibited by the BDD-derived carboxylic acid-CoA esters depleting cellular CoA, inhibiting the enzymes involved in the conjugation of benzoic acid with CoA, or some combination of CoA depletion and enzyme inhibition. Evidence for this hypothesis was obtained by investigating the effect of BDD treatment on the conjugation of benzoic acid with glycine. The significant increase in the ratio of benzoic acid to hippuric acid in rats treated with 1420 or 2272 μmol kg⁻¹ BDD (Fig. 4) is likely the result of carboxylic acids forming CoA esters that in turn inhibit benzoic acid conjugation.

The observed species differences in the inhibitory effect of BDD on benzoic acid conjugation may be related to differences in the ability of rats and mice to conjugate benzoic acid with glycine (Seymour et al., 1987). Because of their lower capacity to conjugate benzoic acid with glycine, rats may be inherently more susceptible to chemically induced inhibition of hippuric acid formation in comparison with mice. The formation of carboxylic acids and the subsequent inhibition of hippuric acid formation may be of toxicological significance. Carboxylic acids can interfere with glucose synthesis and mitochondrial function (Sherratt, 1985; Brass, 1986). Also, elevated levels of benzoic acid can interfere with urea synthesis and cause ammonia-related toxicity (Maswoswe et al., 1986; O’Connor et al., 1987).

Mice and rats exhibited significant differences in the elimination of BDD after administration of equal doses (Fig. 3). Of particular interest, only rats exhibited significant differences in the ratio of benzoic acid to hippuric acid after administration of 1420 μmol kg⁻¹ despite excreting significantly more of the dose as BDD compared with mice receiving the same dose. This suggests that the rat specific inhibition of hippuric acid formation is occurring despite rats eliminating a higher amount of the dose relative to mice. A similar trend was seen when comparing rats dosed with 2272 μmol kg⁻¹ BDD and mice administered 2840 mg kg⁻¹ BDD.

In summary, male B6C3F1 mice and Sprague-Dawley rats were dosed with BDD, a significant human in vitro metabolite of BD. Doses of 1420 and 2272 μmol kg⁻¹ inhibited the conjugation of benzoic acid with glycine in the rat. Mice and rats also excreted the BDD metabolites, crotonic acid, propionic acid, 2-ketobutyric acid, and HBO. Although the amounts of the acids and HBO detected in the urine were low in terms of percentage of dose, their formation provides insight about pathways of BDD metabolism in vivo and the potential formation of the toxic metabolite, crotonaldehyde. Collectively, the results provide further characterization of BDD metabolism in rats and mice and illustrate species differences in BDD disposition. The potential role of BDD and its newly identified metabolites in BD toxicity warrants further investigation. Since BDD formation is expected to be a major pathway of BD metabolism in humans, the carboxylic acids identified in this present study may also be detected in the urine of humans exposed to BD.
Acknowledgments. We thank Renee J. Krause of University of Wisconsin, Department of Comparative Biosciences, for technical discussion throughout the study.

References


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