Methyl-Donor Deficiency Due to Chemically Induced Glutathione Depletion

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ABSTRACT

Dietary deficiency of methionine (Met) is known to deplete cellular Met and cause DNA hypomethylation, but depletion of Met and impairment in methylation due to chemically induced glutathione (GSH) depletion has escaped recognition. In this study, the effect of GSH depletion on the Met pool and methylation capability was examined after bromobenzene (BB), a model GSH-depleting hepatotoxin, was administered to the Syrian hamster. An i.p. dose of BB (800 mg/kg) caused a rapid and extensive depletion of liver GSH; approximately 68% of the initial concentration was depleted during the first hour. The lowest level of GSH, only 4% of the control, was detected at 5 h. GSH depletion was accompanied by a prompt increase in liver Met during the first hour. This initial increase was followed by an extensive depletion during the next 4 h. At 5 h after BB, liver Met was 12% below the control value, and it remained around this concentration throughout the 24-h experiment. To further confirm these results, the endogenous Met pool was labeled with deuterated Met. The administration of L-Met-methyl-d₃ to the Syrian hamster after GSH had been depleted by BB resulted in a significant protection of the liver against necrosis. The protection was accompanied by a marked incorporation of deuterated Met into the liver Met pool. The incorporation, which was determined by gas chromatography-mass spectrometry, shows BB dose dependence. Approximately 53% of the liver Met was labeled when a toxic BB dose (800 mg/kg) was used, while only 25% incorporation was found for the nontoxic dose (100 mg/kg). These results were different from the controls, where only 15% incorporation was found. The differences in the incorporation indicate that there are differences in the degree of utilization and/or depletion of Met in these hamsters, and these differences apparently are dependent upon the degree of toxicity and GSH depletion. The marked incorporation of deuterated Met in the high-dose group was accompanied with a striking increase in the methylation capability. Urinary excretion of the O- and S-methylated 4- and 5-bromo-2-hydroxythiophenols and S-methylated 4- and 5-bromo-2-hydroxy-1,2-dihydrobenzenethiols was significantly increased when compared with the BB treated alone. Approximately 40–45% of the methyl groups in these methylated BB metabolites were methyl-d₃. These results provide direct evidence that depletion of GSH leads to Met depletion and also impairs the methylation processes.

INTRODUCTION

Met³ is known as an essential nutrient for the normal growth and development of mammals (1). Deficiency of this methyl-donor, achieved in animal models fed Met-deficient diets, is known to induce DNA fragmentation and DNA strand breaks (2). Met depletion is also known to lower the threshold of chemical-induced toxicity, and numerous studies have shown that deficiency of methyl-donors is a significant factor in carcinogenesis in rodents (2–12). The precise mechanism(s) of carcinogenesis due to the deprivation of this nutrient is not known, but DNA hypomethylation has been postulated as a cause of neoplastic lesions.

The interrelationship between Met, folates, and GSH is shown in Fig. 1. It is well established that Met and folates are interrelated in the maintenance of one-carbon metabolism (1, 13–21). Under normal conditions, folates supply a significant amount of the methyl group for the resynthesis of Met from homocysteine; this is because the diet generally does not provide enough methyl groups that are needed for cellular methylation reactions (1). It is also known that the intracellular concentration of Met plays a dominant role in determining the metabolic priority of folates (10, 17). When Met is low, folates are diverted away from DNA synthesis to the resynthesis of Met. For the relationship between Met and GSH, it has been established by Reed and Orrenius (22) and Beatty and Reed (23) that the sulfur atom of cysteine and GSH is derived from Met. When GSH is depleted, Met and cysteine are known to contribute equally to the resynthesis of GSH. The effect of this contribution on the cellular content of Met and methylation capability, however, are not known.

Drugs or xenobiotics including chemotherapeutic agents that are metabolized through conjugation with GSH will lead to GSH depletion when a high dosage is used. GSH depletion is generally associated with an increase in toxicity, and the deficiency of GSH for the conjugation reaction is thought to be responsible for such effect. The inhibition of GSH synthesis by buthionine sulfoximine, which also leads to GSH depletion, is known to sensitize tumor cells to chemotherapy and radiation treatment (24, 25). There is no reason to doubt that GSH depletion is involved, but the deficiency of GSH is not necessarily the direct cause of toxicity. The consequences that follow GSH depletion, specifically the metabolic processes leading to GSH turnover, may be more important than the deficiency of GSH per se. When GSH is depleted, the metabolic regeneration of GSH through the sequence of reactions, methionine→cystathionine→cysteine→GSH (Fig. 1), is known to be fully activated (22, 23). This metabolic process should lead to Met depletion when Met intake is limited. Evidence for such a Met depletion came from the finding in our laboratory (26) that liver necrosis caused by BB, a GSH-depleting hepatotoxin, is associated with an impairment in basic biological methylation in the male golden Syrian hamster. A single i.p. dose of BB (800 mg/kg) caused severe liver and kidney necrosis within 24 h. The appearance of necrosis was accompanied by an impairment in the methylation of the 4- and 5-bromo-2-hydroxythiophenols (bromothiocatechols) and 4- and 5-bromo-2-hydroxy-1,2-dihydrobenzenethiols (bromodihydrobenzenethiolols). The methylation process was repaired by the administration of a single i.p. dose (1200 mg/kg) of NAM at 5 h after BB. This led to a significant decrease in toxicity with a concomitant striking increase in urinary excretion of the O- and S-methylated bromothiocatechols and S-methylated bromodihydrobenzenethiolols. Based on these findings (26), we have postulated a new hypothesis for the mechanism of BB-induced toxicity. We suggested that Met depletion resulting from GSH depletion is directly responsible for BB toxicity.

The present study was designed to test the hypothesis that depletion of hepatic GSH will lead to Met depletion. BB was used as a model GSH-depleting hepatotoxin in this study. GSH and Met time-response curves were examined after BB was administered to Syrian hamsters.
Liver GSH was determined by a general GSH assay (27), while the concentration of Met in the liver was determined by GC-MS. L-Met-methyl-d$_3$ was used as an internal standard, and the quantitation of Met was carried out by selected ion detection of the AC-TBDMS derivatives. To further confirm that the Met pool was indeed depleted, the endogenous Met pool was labeled with deuterated Met. L-Met-methyl-d$_3$ was administered to hamsters after GSH had been depleted by BB, and the incorporation of deuterated Met into the endogenous Met pool was examined by GC-MS. The degree of incorporation was compared between hamsters that were treated with a high- (800 mg/kg) and nontoxic (100 mg/kg) dose of BB, and these results were used to compare with those observed from the controls that received only deuterated Met. The effect of L-Met-methyl-d$_3$ on the methylation capability was also examined for the incorporation of methyl-d$_3$ into the O- and S-methylated bromothiocatechol and S-methylated bromodihydrobenzenethiol metabolites of BB.

**MATERIALS AND METHODS**

**Materials**

Reference compounds were obtained from the following sources: bromobenzene (Aldrich Chemical Co., Milwaukee, WI); acetic anhydride, heparin, reduced glutathione, 5,5'-dithio-bis(2-nitrobenzoic acid), and 5-sulfosalicylic acid (Sigma Chemical Co., St. Louis, MO); l-methionine-methyl-d$_3$ (98% deuterium content; Cambridge Isotope Laboratories, Woburn, MA); Glusulase (DuPont/New England Nuclear Research Prod-
ucts, Boston, MA); and bis(trimethylsilyl)acetamide and N-methyl-N-( tert-butyldimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, IL). All other reagents were of the highest grade commercially available.

**Animal Treatments**

Young adult male golden Syrian hamsters were obtained from Charles River Laboratories (Wilmington, MA). Animals were 32-40 days of age and weighed between 80 and 100 g. Animals were housed in groups of four on a bedding of hardwood shavings. They were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Hamsters were allowed to acclimate in the animal facility for 4–5 days before use. They had free access to food (Purina Lab Chow) and water.

**Effect of BB Toxicity on Liver Glutathione and Methionine**

Following the acclimation period, a group of hamsters was treated with a single i.p. dose of BB (800 mg/kg, 5.12 mmol/kg, and 0.54 ml/kg) in corn oil (0.2 ml). Animals were housed on a bedding of hardwood shavings in shoe-box plastic cages. Animals received no food but had free access to drinking water. At specific time intervals as indicated in Fig. 2, hamsters were anesthetized with ether. Blood was drawn by cardiac puncture into a heparin-treated syringe, and plasma was separated after centrifugation. The extent of liver damage was evaluated by measuring plasma GPT activity using Sigma kit 505. Livers were excised, and gall bladders were removed. The livers were rinsed with ice-cold saline, patted dry, and quickly weighed. The same section from each liver was sliced and fixed in 10% buffered neutral formalin solution for histological examination. The rest of the liver was again weighed, frozen in liquid nitrogen, and stored at −70°C for GSH and Met determination. For controls, these hamsters received no BB, but an i.p. injection of corn oil (0.2 ml) was given. Identical procedures as those described above were used.

**L-Methionine-Methyl-d3 Labeling Experiments**

For the high BB dose group, hamsters were divided into two major groups after the acclimation period. The first group (BB) was treated with an i.p. injection of BB (800 mg/kg) in corn oil (0.2 ml) and followed with a 1-ml injection of distilled water 5.5 h later. The second group (BB + Met) was dosed with BB as described, but an i.p. injection of L-Met-methyl-d3 (450 mg/kg, 2.96 mmol/kg) in 1 ml of distilled water was given 5.5 h after BB. Each hamster was housed in a metabolism cage, and a 0–24-h urine sample was collected. Animals received no food but had free access to drinking water during the 0–24 h urine collection. Urine samples were stored at −20°C until analyzed.

At 24 h after BB, hamsters were anesthetized with ether. Plasma samples were collected, and the extent of liver damage was evaluated. Livers were excised and treated as described. For the low BB dose group, a lower dose of BB (100 mg/kg, 0.64 mmol/kg, 0.07 ml/kg) was given to another set of hamsters after the acclimation period. Identical methods as those described for the high BB dose group were used. For controls, these hamsters received no BB, but an i.p. injection of corn oil (0.2 ml) was given and followed with an i.p. injection of L-Met-methyl-d3 in 1 ml distilled water 5.5 h later. Identical procedures as those described above were used.

**Analyses of Urinary Metabolites**

Urinary metabolites of BB obtained from hamsters that were dosed with BB and L-Met-methyl-d3 were examined specifically for the incorporation of methyl-d3 into: (a) O- and S-methylated bromothiocatechols; (b) S-methylated bromodihydrobenzenethiolols; and (c) 3- and 4-methoxy-bromocatechols. Briefly, an aliquot of the 0–24-h urine sample (usually one-tenth of total volume) was hydrolyzed with Glusulase (9000 units glucuronidase and 1000 units sulfatase), and neutral/phenolic metabolites were isolated by the amino acid, and after centrifugation (5000 rpm for 5 min), an aliquot of the supernatant was diluted with a 0.1 M potassium phosphate buffer (pH 8.0), and 100 μl of 5.5'-dithio-bis(2-nitrobenzoic acid) (10 mM, pH 7.4) was added for the determination of total liver GSH. Briefly, an aliquot (usually 500 μl) was deproteinized with an equal volume of 4% sulfosalicylic acid, and after centrifugation (5000 rpm for 5 min), an aliquot of the supernatant was diluted with a 0.1 M potassium phosphate buffer (pH 8.0), and 100 μl of 5.5′-dithio-bis(2-nitrobenzoic acid) (10 mM, pH 8.0) were added. The total volume of each sample was 1 ml. Reduced glutathione was proportional to the absorbency at 412 nm.

**Isolation and Quantification of Liver Methionine**

Liver Met was converted to the N-acetyl derivative by a modification of published methods (29, 30). Briefly, a known amount of L-Met-methyl-d3 was added to 250 μl of liver homogenate to serve as an internal standard for Met. For the L-Met-methyl-d3 labeling experiments, no internal standard was added. The mixture was cooled in a beaker of ice while 100 μl glacial acetic acid were added; then samples were extracted with ethyl acetate. Following the extrac-
tion, the aqueous phase was frozen in an acetone-dry ice bath and lyophilized. A 0.5-ml aliquot of glacial acetic acid was added to the dry residue, vortexed, and allowed to stand at room temperature for 5 min before the addition of 10 μl of acetic anhydride. The acetylation reaction was allowed to proceed at room temperature for 30 min. At the end of the reaction period, excess acetic anhydride in the sample was removed by reacting with an excess volume of absolute ethanol. This reaction converts acetic anhydride to ethyl acetate. The sample was centrifuged, and the clear solution was transferred to a small round bottom flask and dried by Roto-evaporation. The residue was dissolved in distilled water and transferred to a centrifuge tube. The pH of the solution was adjusted to 2–3 with 1 N sulfuric acid, and the sample was extracted twice with ethyl acetate. Extracts were combined and concentrated under a gentle stream of nitrogen to a small volume for storage and derivative formation for analyses by GC and GC-MS.

GC and GC-MS Analyses

Preparation of Derivatives. For urinary metabolites, an aliquot of the neutral/phenolic extract (usually one-fifth of the total extract) was used for derivative formation. These metabolites were converted to TMS derivatives as described (31).

For liver methionine, the ethyl acetate extract was evaporated by nitrogen in a small screw-capped glass vial, and 20 μl of pyridine was added to dissolve the dry residue. Following the addition of 30 μl of N-methyl-N-(tert-butyldimethyl)silyl)trifluoroacetamide, the reaction mixture was heated at 60°C for 30 min. GC and GC-MS studies were carried out with 0.1- to 0.2-μl samples.

GC Analyses. Analyses were carried out with a Hewlett Packard 5890 gas chromatograph equipped with a flame-ionization detector. A 30-m (0.32-mm inside diameter, 0.25-μm film thickness) fused silica DB-5 column (J & W Scientific, Folsom, CA) was used. All GC analyses were temperature programmed from 60°C to 300°C at the rate of 2°C/min.

GC-MS Analyses. Analyses of urinary metabolites were carried out with a Nermag R10–10C (original Delsi Nermag Instrument, Houston, TX) mass spectrometer coupled to a Varian 3400 gas chromatograph (Varian Instrument, Walnut Creek, CA). A PDP 11/73 data system (Digital Equipment Corporation, Bedford, MA) was used. The mass spectrometry analyses were carried out in an electron impact ionization mode. A 30-m (0.32-mm inside diameter, 0.25-μm film thickness) fused silica DB-5 capillary column was used. The ion source temperature was 200°C, and the electron energy was 70 eV. Helium was used as the carrier gas. The separation was carried out with temperature programming from 100°C to 300°C at 5°C/min. GC and GC-MS properties (TMS derivatives) of all the neutral/phenolic metabolites of BB have been described previously (31, 32).

GC-MS Analyses of liver methionine were carried out by both full scan and selected ion detection using a Fisons Quattro mass spectrometer (Fisons VG Biotech, Altrintham, United Kingdom) coupled to a Hewlett Packard 5890 gas chromatograph. An Intel 486-based PC with Fisons Masslynx software was used. The mass spectrometry analyses were carried out in an electron impact ionization mode. A 30-m (0.32 mm inside diameter, 0.25-μm film thickness) fused silica DB-1 capillary column was used. The ion source temperature was 200°C, and the electron energy was 70 eV. Helium was used as the carrier gas. The separation was carried out with temperature programming from 150°C to 300°C at 10°C/min.

Statistical Analysis. One-way ANOVA (GraphPAD InStat, San Diego, CA) was used to determine the statistical significance of the incorporation of L-Met-methyl-d3 into the liver Met pool.

RESULTS

Effect of BB Toxicity on Liver Glutathione and Methionine. We have reported previously (26) that the administration of a toxic dose of BB (800 mg/kg) to the Syrian hamster resulted in severe liver necrosis with very high plasma GPT activity at 24 h. When the time-response curve of plasma GPT activities was examined in the present study, it was found that GPT activity was not significantly elevated until after the 11-h time point (Fig. 2A). A slow but steady elevation of GPT activity was observed after 5 h, and a mild degree of fatty liver was found in some hamsters. After the 11-h time point, GPT activity increased rapidly in all animals. By 15 h after dosing, some hamsters had developed a mild degree of intrabiliary hemorrhage. Severe necrosis with massive hemorrhage was found in all hamsters at 24 h.

Several interesting results were found when liver GSH was determined at these time points. The GSH time-response curve in the Syrian hamster (Fig. 2B) shows similar characteristics to that found in an earlier study using the Sprague-Dawley rat (27). A rapid and extensive depletion of liver GSH was observed during the first hour. Approximately 68% of the initial liver GSH was depleted during this hour. During the next 4 h (2–5 h), GSH levels continued to decline but at a much slower rate than occurred during the first hour. This decline in the rate of depletion may due to the initiation of GSH turnover, resulting in newly synthesized GSH compensating for GSH depletion (33–35). Liver GSH reached the lowest level around 5 h after BB; this level was only 4% of the initial GSH concentration. Although the exact time point when GSH resynthesis is initiated is not clear from this time-response curve, the regeneration of GSH was clearly evident after the 5-h time point. At 24 h, liver GSH returned to 40% of the initial, pre-treatment value. It is interesting to note that the resynthesis of liver GSH (Fig. 2B) occurred in parallel with the elevation of GPT activity (Fig. 2A).

Fig. 3 shows a typical GC-MS analysis by selected ion detection of liver methionine as AC-TBDMS derivatives. The bottom chromatographic chart shows the total ion current, while the top and the middle charts show ion detection at m/z 248.1 and 251.2 amu, corresponding to the [M-57]+ ion for d3-Met and d3-Met (AC-TBDMS derivatives), respectively. The area under the peaks was used for quantitative determination of endogenous Met. The time response curve shown in Fig. 2C was obtained from these calculations.

Fig. 4 shows the mass spectra (AC-TBDMS derivatives) of d3-Met (top panel) and d3-Met (bottom panel). A strong molecular ion was observed for the d3-Met at m/z 305.2. This molecular ion increased 3 amu to m/z 308.3 when d3-Met was used. The base peaks in these mass spectra were present at m/z 248.1 (for d3-Met) and 251.2 (for d3-Met) and were due to the loss of C(CH3)3 (M-57) from their respective molecular ions. This is a typical characteristic kind of mass fragmentation for the TBDMS derivative. These ions were used for selected ion detection in Fig. 3. An intense ion was present at m/z 231.2 in both mass spectra. This ion, which corresponds to M-74 in the top mass spectrum, was due to the loss of CH2=CH-S=CH2 from the molecular ion (m/z 305.2), while it was due to the loss of 77 amu (CD3SCH=CH2) from m/z 308.3 in the bottom mass spectrum. Another intense ion at m/z 200.1 (Fig. 4, top) and 200.2 (Fig. 4, bottom) was due to the loss of HSCH3 from m/z 248.1 (Fig. 4, top) and HSCD3 from m/z 251.2 (Fig. 4, bottom). As indicated by the methylene unit values (28), the d3-Met had an earlier GC retention time than the d3-Met (Fig. 3).

Fig. 2C shows the time response curve of liver Met after BB administration. The rapid and extensive depletion of GSH during the first hour time point (Fig. 2B) was accompanied by a prompt increase in liver Met (Fig. 2C). A 17% increase from the control value was found at the first hour time point. Under the circumstance where Met intake is limited, as in this instance, an increase in the concentration of Met indicates that the resynthesis of Met by liver cells has occurred.

Liver Met declined after the first hour; a sharp decrease was observed at the third hour time point. By 5 h after BB, liver Met was 12% below the control level. Throughout the 24-h period, liver Met never regained its normal value but remained below the basal level.

Prevention of BB Toxicity by L-Methionine-Methyl-d3. Liver toxicity caused by a single i.p. dose of BB in the golden Syrian hamster is dose dependent. Fig. 5 shows a comparison of the effect of BB doses on the gross liver appearances at 24 h. A high dose of BB
METHYL-DONOR DEFICIENCY DUE TO GSH DEPLETION

The administration of a single i.p. dose (450 mg/kg) of L-Met-methyl-d₃ to the Syrian hamster at 5.5 h after BB resulted in a significant decrease in toxicity in the high-dose group, but no effect was found in the low-dose group (Figs. 5 and 6). Intrahepatic hemorrhage, which is a typical characteristic of liver necrosis in the high-dose group, was no longer observed in the L-Met-methyl-d₃-treated animals. Hepatocellular swelling, however, remained clearly evident by gross liver appearances (Fig. 5). Histological examination revealed specific protection (Fig. 6D) by Met; a moderate diffuse cellular swelling was still observed. The degree of protection by Met in these hamsters showed similar characteristics to those observed in our previous study (26) using NAM as an antidote.

L-Methionine-Methyl-d₃ Labeling Experiments. The administration of deuterated Met to hamsters after GSH was depleted by BB allowed us to study the effect of BB on the endogenous liver Met pool more closely. These studies were carried out by GC-MS procedure. Regardless of the BB dosage used, the administration of d₃-Met at 5.5 h after BB always resulted in an incorporation of d₃-Met into the endogenous Met pool at 24 h. The degree of incorporation, however, was strikingly different and appears to be highly dependent upon the BB dosage used (Fig. 7). At 24 h after BB, approximately 53 ± 20% of liver Met was labeled in the high-dose group, whereas only 25 ± 4% was d₃-Met in the low-dose group. These results are different from those observed in control hamsters in which only 15 ± 6% of the liver Met was labeled.

When the urinary metabolites of BB from the high and low BB dose group were examined, a striking result was found in terms of the incorporation of methyl-d₃ from L-Met-methyl-d₃ into O- and S-methylated metabolites of BB. In the high-dose group, significant amounts of the methyl-d₃ were incorporated into the methyl group of the four isomeric O- and S-methylated bromothiocatechols (Fig. 8), whereas no incorporation was found in the low-dose group (data not shown). Each of these metabolites, which generally appeared as single sharp chromatographic peaks in the nonlabeled hamsters (26), now appeared as twin chromatographic peaks in the Met-methyl-d₃-treated animals (Fig. 8). GC-MS analyses of the TMS derivatives of these twin chromatographic peaks indicated that they are a mixture of methyl-d₃ and methyl-d₀ O- and S-methylated bromothiocatechols. Partial mass spectra (TMS derivatives) of a twin chromatographic

(800 mg/kg) caused severe liver necrosis with massive intrahepatic hemorrhage (Fig. 6C). This result is consistent with those found in our previous study (26). In the present study, the administration of a single low dose of BB (100 mg/kg) to the Syrian hamster did not produce a comparable degree of toxicity. In fact, no necrosis or hemorrhage was found in these animals. The gross liver appearances in the low-dose group were normal (Fig. 5), and biochemical determination indicated normal GPT activities (data not shown). Histological examinations by light microscopy confirmed these results (Fig. 6A).

Fig. 3. GC-MS analysis by selected ion detection of liver Met as AC-TBDMS derivatives. L-Met-methyl-d₃ was used as an internal standard for the quantification of liver Met. Bottom, scan of total ion current of d₀- and d₃-Met. Top and middle, scans of ions at m/z 248.1 and 251.2 amu, corresponding to the [M-57]+ ion for d₀- and d₃-Met, respectively.

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peak with a retention time of 44.3 min in Fig. 8 is shown in Fig. 9 (top and middle mass spectra). A partial mass spectrum (TMS derivative) of the same chromatographic peak, but obtained from a nonlabeled animal, is shown in the bottom panel for comparative purposes. The bottom mass spectrum, which showed a molecular ion at m/z 290, has been identified previously as one of the four isomeric O- and S-methylated 4- and 5-bromo-2-hydroxythiophenols (32). The middle mass spectrum in Fig. 9, which is nearly identical to the bottom mass spectrum, also showed a strong molecular ion at m/z 290; this mass spectrum contained mainly methyl-d0 methylated bromothiocatechol.
For the top mass spectrum, two distinct molecular ions were clearly present at $m/z$ 290 and 293. The molecular ion at $m/z$ 293, which is 3 amu higher than $m/z$ 290, is the methyl-$d_3$ methylated bromothiocatechol. The base peak at $m/z$ 260 was shared by both the methyl-$d_0$ and methyl-$d_3$ methylated bromothiocatechols. This ion is due to consecutive losses of two methyl groups (M-CH$_3$-CH$_3$) from $m/z$ 290, whereas for methyl-$d_3$ methylated product, it is due to the loss of M-CH$_3$-CD$_3$ from $m/z$ 293. Based on mass spectrometric analyses, the amount of methyl-$d_3$ incorporated into each methylated bromothiocatechol in Fig. 8 was approximately 40-45%.

Similar results were also found for the incorporation of methyl-$d_3$ from L-Met-methyl-$d_3$ into the two isomeric S-methylated 4- and 5-bromo-2-hydroxy-1,2-dihydrobenzenethiols and also to the 3- and 4-methoxybromocatechols (data not shown).

**DISCUSSION**

**Relationship between Liver Glutathione and Methionine.** It has been our view that BB-induced toxicity is associated with Met depletion, a depletion which we have postulated to occur as a result of GSH depletion (26). Following BB administration, a rapid and extensive depletion of liver GSH was observed during the first hour time point (Fig. 2B). GSH depletion was accompanied by a prompt increase in liver Met (Fig. 2C). This rapid responsiveness of the liver indicates a direct relationship between GSH and Met. In the metabolic pathway in Fig. 1, Met can be generated through transmethylation of homocysteine; homocysteine must acquire a methyl group to complete this reaction (1, 13-21). Our current observation (36) suggests that the methyl group could be derived from 5-methyltetrahydrofolate (discussed below). This suggestion, however, did not exclude the possibility that some Met could also be derived from protein turnover. Besides transmethylation, homocysteine is also metabolized via the transsulfuration pathway in which cysteine and GSH are generated. The direction of the metabolism of homocysteine is known to be regulated by SAM (1, 16, 21). When the level of Met/SAM is high in the liver, the transsulfuration pathway is favored. However, under the condition when the level of Met/SAM is low, homocysteine methylation is promoted. The observation of a prompt increase in liver Met after BB administration provides a direct indication that the level of Met/SAM is low in the liver.

The regeneration of Met (Fig. 2C) also restores the transsulfuration pathway of metabolism of homocysteine. This metabolic process leads to cysteine and GSH turnover (Fig. 2B). The continuation of the utilization of GSH by BB metabolite(s), however, leads to a continuation of the utilization of Met. Under the circumstance where Met
intake is limited, this process leads to Met depletion. A depletion of Met following an extensive resynthesis is presumably due to an exhaustion of methyl-donor for transmethylation of homocysteine; extensive utilization of a methyl-donor(s) would lead to its depletion.

The administration of d₃-Met after GSH was depleted by BB allowed us to examine the effect of GSH depletion on the endogenous Met pool more closely. Significant differences in the degree of incorporation of d₃-Met into the endogenous liver Met pool were found between the high and low BB dose group (Fig. 7). At 24 h after BB, approximately 53 ± 20% of the endogenous liver Met was replaced by exogenous d₃-Met in the high-dose group, whereas only 25 ± 4% was labeled amino acid in the low-dose group. These differences in the incorporation indicate that there are differences in the degree of utilization and/or depletion of Met in these hamsters, and these differences apparently are dependent upon the degree of toxicity and GSH depletion.

We have reported in a previous study (26) that the toxicity of BB is associated with impairment in O- and S-methylation of 4- and 5-bromo-2-hydroxythiophenols in the Syrian hamster. When these BB metabolites were examined in the d₃-Met-treated hamsters, several interesting results were found. A marked incorporation of methyl-d₃ into O- and S-methylated bromothiocatechols (Figs. 8 and 9) was found in the animals that were treated with a high dose of BB. Approximately 40—45% of methyl-d₃ was found in each methylated thiol-containing metabolite shown in Fig. 8. Interestingly, this incorporation was only observed in hamsters that were treated with a high dose of BB, while no incorporation was found in the low-dose group. These observations are different from those found when endogenous liver Met was examined directly. In those experiments, the incorporation of d₃-Met was found in both groups at 24 h after BB (Fig. 7). These results suggest that the formation of bromothiocatechols (potential toxic metabolites of BB (26, 31, 32, 37)) must have occurred before the administration of d₃-Met; d₃-Met was not administered until 5.5 h after BB. In the low-dose group, these hamsters showed no sign of toxicity (Figs. 5 and 6A). When bromothiocatechols were
formed, these metabolites could promptly be methylated by endogenous SAM. This resulted in 100% formation of methyl-d$_0$ O- and S-methylated bromothiocatechols in these hamsters. For the high toxic dose group, data from the methylated bromothiocatechols shown in Figs. 8 and 9 indicate that part of these metabolites were able to be methylated by endogenous SAM, but approximately 40–45% were not methylated. A low level of endogenous Met and SAM would have led to such an observation. The administration of exogenous deuterated Met to these hamsters led to a significant decrease in toxicity and provided an additional methyl-d$_3$-labeled SAM for the methylation of the rest of the bromothiocatechols. This resulted in a mixture of methyl-d$_0$ and methyl-d$_3$ methylated products.

Results from the isotope labeling experiments are consistent with an earlier finding from a study of naphthalene metabolism in the rat where deuterium labeling of methylthio metabolites of naphthalene was only observed when animals were maintained on a methionine-free diet for 7 to 8 days before supplementation of the diet with d$_3$-Met; no incorporation was found when animals were maintained on a normal diet.

**Relationship between BB Toxicity and the Content of Liver Glutathione and Methionine.** It has generally been accepted that GSH deficiency is the direct cause that elicits BB toxicity (27). This early postulation was based mainly on the finding that the threshold of toxicity was decreased when the animal was pretreated with diethylmaleate, while the administration of the GSH precursor, cysteine, decreased the toxicity. This assumption was made before the recognition that GSH depletion also leads to Met/SAM depletion. The observation that NAM could provide a striking protection of the liver against BB-induced necrosis while the resynthesis of GSH was inhibited by propargylglycine, an irreversible inhibitor of cystathionase (23), provides direct evidence that methyl-donor deficiency plays a significant role in eliciting toxicity.

Furthermore, the late onset of toxicity is not well correlated with GSH depletion (Fig. 2); GSH depletion occurred far in advance of the elevation of GPT activity. GSH depletion at an early time point could reflect a depletion of the GSH from the cytosolic pool. It has been shown that the cytosolic pool of GSH could be depleted without affecting cell viability, whereas depletion of the mitochondrial pool is a necessary event for the expression of cytotoxicity (39, 40). The effect of BB on these separate pools of GSH was not investigated in greater detail in the present study. It is interesting to note that the elevation of liver toxicity was parallel to the regeneration of GSH (Fig. 2). This result suggests that the metabolic process leading to GSH turnover could be responsible for liver injury. The ability of the hepatic cell to regenerate cysteine and GSH when food intake is limited must occur through the expense of other available nutrient(s). In this instance, these other nutrients are Met and SAM.

In the metabolic pathway in Fig. 1, the resynthesis of Met through transmethylation of homocysteine would divert tetrahydrofolate from the biosynthesis of the pyrimidine, thymidylate. This would lead to an impairment in normal DNA synthesis and repair processes. Our current observation (36) is consistent with this hypothesis. When a single i.p. dose of thymidine (200 mg/kg) labeled with [2-¹⁴C]thymidine (5 μCi) was given to Syrian hamsters at 5 h after BB, the result was striking. Intrahepatic hemorrhage, which is a typical characteristic of liver necrosis in the Syrian hamster, was significantly decreased in the thymidine-treated animals. Liver toxicity, however, remained clearly evident, as indicated by plasma GPT activity and histological examination. When DNA was isolated, the BB- and thymidine-treated hamsters showed approximately a 2-fold increase in thymidine incorporation when compared with those treated with thymidine alone. This result suggests that BB toxicity is associated with DNA damage, a damage that arises from Met/SAM depletion.

In conclusion, the present study provides direct evidence that there is a tight interrelationship between liver GSH and Met; therefore, agents that deplete GSH will deplete Met. Although Met depletion resulting from dietary deficiency of Met is a well-recognized phenomenon, Met depletion arising from GSH depletion has escaped recognition. The biological significance arising from GSH depletion is that it causes an impairment in methylation. This impairment is now known to have a marked impact on the methylation of DNA; significant DNA hypomethylation was found in the high BB dose group.

Fig. 9. Partial mass spectra (TMS derivatives) of methylated bromothiocatechol. Top and middle are mass spectra of a twin chromatographic peak shows in Fig. 8 (retention time, 44.3 min). The twin chromatographic peak is a mixture of methyl-d₃ (m/z 293) and methyl-d₀ (m/z 290) methylated bromothiocatechol. A pure partial mass spectrum (TMS derivative) of methyl-d₀ methylated bromothiocatechol (m/z 290), which was obtained from a nonlabeled hamster, is shown in the bottom panel for comparative purposes. MU, methylene unit.

Met depletion and impairment in methylation are prominent biochemical changes which can and will occur with a variety of toxins and chemotherapeutic agents involving GSH conjugation as part of their metabolic transformation. Due to the crucial role of Met in many basic biological reactions, Met depletion would lead to a variety of effects, including altered protein synthesis and gene expression. An early observation that diethylmaleate inhibits protein synthesis in the liver and brain of mice (41) could also be due to Met depletion; protein synthesis specifically requires Met for the initiation of translation. For BB, we believe that the cause(s) of toxicity is not limited only to the formation of toxic metabolite(s) but rather results from a combination of effects arising from biological changes in basic housekeeping processes due to Met depletion and impairment in methylation in conjunction with toxic metabolite(s) formation. An alteration of DNA processes due to Met depletion and impairment in methylation, together with an impairment of normal DNA synthesis and repair, could be the initial factor that elicits toxicity in response to a variety of GSH-depleting agents, including those modeled here by BB.

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Methyl-Donor Deficiency Due to Chemically Induced Glutathione Depletion

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