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Alcohol Dehydrogenase- and Rat Liver Cytosol-Dependent Bioactivation of 1-Chloro-2-hydroxy-3-butene to 1-Chloro-3-buten-2-one, a Bifunctional Alkylating Agent

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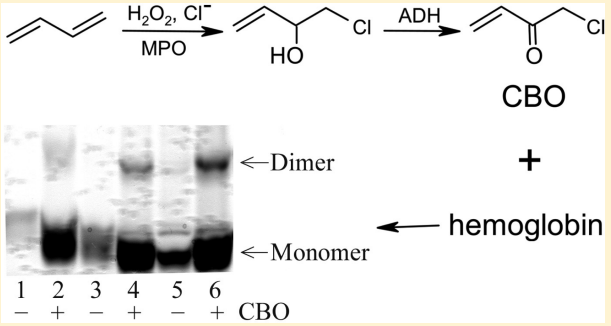
ABSTRACT: 1,3-Butadiene (BD) is an air pollutant whose toxicity and carcinogenicity have been considered primarily mediated by its reactive metabolites, 3,4-epoxy-1-butene and 1,2,3,4-diepoxybutane, formed in liver and extrahepatic tissues by cytochromes P450s. A possible alternative metabolic pathway in bone marrow and immune cells is the conversion of BD to the chlorinated allylic alcohol 1-chloro-2-hydroxy-3-butene (CHB) by myeloperoxidase in the presence of hydrogen peroxide and chloride ion. In the present study, we investigated the in vitro bioactivation of CHB by alcohol dehydrogenases (ADH) under in vitro physiological conditions (pH 7.4, 37 °C). The results provide clear evidence for CHB being converted to 1-chloro-3-buten-2-one (CBO) by purified horse liver ADH and rat liver cytosol. CBO readily reacted with glutathione (GSH) under assay conditions to form three products: two CBO-mono-GSH conjugates [1-chloro-4-(*S*-glutathionyl)butan-2-one (**3**) and 1-(*S*-glutathionyl)-3-buten-2-one (**4**)] and one CBO-di-GSH conjugate [1,4-*bis*(*S*-glutathionyl)butan-2-one (**5**)]. CHB bioactivation and the ratios of the three GSH conjugates formed were dependent upon incubation time, GSH and CHB concentrations, and the presence of ADH or rat liver cytosol. The ADH enzymatic reaction followed Michaelis–Menten kinetics with a K_m at 3.5 mM and a k_{cat} at 0.033 s^{-1}. After CBO was incubated with freshly isolated mouse erythrocytes, globin dimers were detected using SDS-PAGE and silver staining, providing evidence that CBO can act as a protein cross-linking agent. Collectively, the results provide clear evidence for CHB bioactivation by ADH and rat liver cytosol to yield CBO. The bifunctional alkylating ability of CBO suggests that it may play a role in BD toxicity and/or carcinogenicity.



INTRODUCTION

1,3-Butadiene (BD) is a major petroleum product used in the production of synthetic rubber and plastic. It is also found in cigarette smoke, gasoline, and urban air.[1] BD has been classified as a human carcinogen by the U.S. Environmental Protection Agency primarily because epidemiological studies showed increased incidences of lymphohematopoietic cancers among workers occupationally exposed to BD.[1]

The carcinogenicity of BD is widely believed to be mediated by its primary and/or secondary cytochromes P450-dependent metabolites (epoxides), namely, 3,4-epoxy-1-butene (EB), 1,2,3,4-diepoxybutane (DEB), and 3,4-epoxy-1,2-butanediol (EBD).[2] While EB, EBD, and DEB are all direct-acting mutagens/carcinogens,[3] DEB is the most potent mutagen among these epoxides,[3−5] possibly due to its higher reactivity and cross-linking ability.[6−8] DEB can induce in vitro/in vivo DNA–DNA[6,7,9−15] and in vitro DNA–protein cross-linking.[16−20] Interestingly, it almost exclusively causes DNA–DNA cross-linking in cells.[21] DEB is widely believed to be the ultimate metabolite responsible for mutagenicity/carcinogenicity of BD,[8,22] although it has been suggested that EBD might play a more important role in rats and humans.[23,24]

Evidence for a possible alternative metabolic pathway involving myeloperoxidase (MPO) has been obtained.[25] BD has been demonstrated to be oxidized to 1-chloro-2-hydroxy-3-butene (CHB, **1**) by isolated human MPO in the presence of H_2O_2 and chloride ion (Scheme 1).[25] The reaction probably occurred through the initial formation of hypochlorous acid (HOCl) by MPO. EB was also detectable in these incubations, but CHB was the major product detected at chloride concentrations >50 mM.[25] Although CHB is unlikely to be produced in liver, lung, or kidney cells for lack of expression of MPO and the extremely low intracellular H_2O_2 concentrations (≤0.1−1 μM by estimation[26]), the MPO pathway is expected to be effective in immune and bone marrow cells, such as neutrophil granulocytes. Neutrophil granulocytes contain large amounts of MPO and high concentrations of chloride ion (80−111 mM)[27] and can produce high concentrations of H_2O_2 (in

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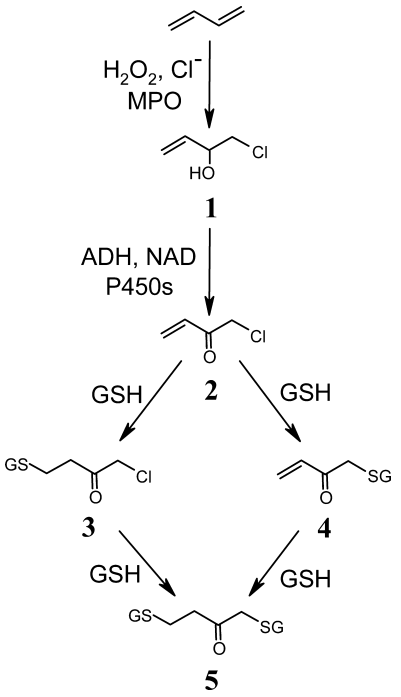
Scheme 1. Metabolic Pathway for the Formation of CHB (1) and CBO (2) from BD and the Subsequent Reactions of CBO with GSH To Form Three CBO−GSH Conjugates (3−5)



millimolar) for microbial killing when the cells are stimulated by invading microorganisms or upon oxidative burst as part of the inflammatory response. HOCl is believed to be the actual agent to kill bacteria.[28] When the chloride ion concentration is higher than 50 mM, more than 90% of H_2O_2 is converted to HOCl.[27] Thus, there should be sufficiently high concentrations of HOCl in neutrophil granulocytes to lead to the formation of CHB when the cells are exposed to BD.

After its formation in vivo, CHB, a halogenated allylic alcohol, is likely to undergo oxidation by alcohol dehydrogenases (ADHs) and cytochromes P450s. Support for this hypothesis is provided by our previous demonstration that 3-butene-1,2-diol (BDD) can be metabolized by ADHs and cytochromes P450 both in vitro and in vivo to yield the Michael acceptor hydroxymethylvinyl ketone (HMVK).[29−33] We postulated that CHB could similarly be transformed to 1-chloro-3-buten-2-one (CBO, **2**) by ADHs (Scheme 1). On the basis of its chemical structure, CBO is expected to act as a bifunctional alkylating agent.

In the present study, we examined the in vitro transformation of CHB to CBO by purified horse liver ADH and rat liver cytosol. Glutathione (GSH) was included in the experiments as a trapping agent. The results provide clear evidence for CHB bioactivation to CBO by ADH and cytosolic ADH from rat liver, and the generated CBO readily reacted with GSH to form two CBO−mono-GSH conjugates (**3** and **4**) and one CBO−di-GSH conjugate (**5**) (Scheme 1). Incubation of erythrocytes with CBO resulted in the detection of globin dimers, providing further evidence for the cross-linking ability of CBO.

EXPERIMENTAL PROCEDURES

Materials. BD (99.5%) was purchased from Matheson Gas (Joliet, IL). Calcium hypochlorite, chromium oxide, perchloric acid, trifluoroacetic acid (TFA), Trizma base, glycerol, sodium dodecyl sulfate, bromophenol blue, 2-mercaptoethanol, DTT, acetonitrile (HPLC grade), purified horse liver ADH (catalog #A9589), nicotinamide adenine dinucleotide (NAD), and GSH were obtained from Sigma-Aldrich (St. Louis, MO). Rat liver cytosol was isolated from male Sprague−Dawley rats as described previously.[34,35] The whole blood of male C57Bl mice was obtained from Harlan Bioproducts (Indianapolis, IN). CHB was synthesized as described previously.[36]

Instruments and Methods. Mass spectra were obtained on an MDS Sciex API 365 LC/MS/MS triple quadrupole electrospray ionization (ESI) mass spectrometer. NMR spectra were recorded on a Bruker Instruments DMX-400 Avance spectrometer (400 MHz). HPLC separation was performed on a gradient-controlled HPLC system (Gilson Inc., Middleton, WI) equipped with a Beckman System Gold 166 variable wavelength detector (San Ramon, CA) using a Beckman Ultrasphere 5 μm ODS reverse-phase analytical column (4.6 mm × 25 cm) with UV detection at 254 nm. A linear gradient program was used, starting at 0 min from 0% pump B to 10% pump B over 7 min [pump A, 1% (v/v) acetonitrile with the pH being adjusted to 2.5 with TFA; pump B, 75% (v/v) acetonitrile at pH 2.5] at a flow rate of 1 mL/min and then at 7 min from 10 to 20% pump B over 1 min, at 12 min from 20 to 10% pump B over 1 min, and stopped at 17 min. The retention times of the three CBO−GSH conjugates under these conditions were 14.7 min for **3**, 9.4 min for **4**, and 7.1 min for **5**. The standard curves were linear with the ranges of quantities and correlation coefficients as follows: **3**, 0.6−484 nmol, 0.9980; **4**, 1.33−106.4 nmol, 0.9968; and **5**, 0.37−292.96 nmol, 0.9948, respectively. For preparative separation, a Beckman Ultrasphere 5 μm ODS semipreparative column (250 mm × 10 mm) was employed. The solvents in pump A and B were 1 and 15% acetonitrile at pH 2.5, respectively. A 35 min isocratic program with 50% pump B was used with UV detection at 220 nm.

Synthesis of CBO. CBO was prepared through oxidation of CHB by Jones' reagent. The reaction was performed following a procedure described in the literature.[37] Jones' reagent was prepared by the addition of concentrated H_2SO_4 (1.0 mL) to CrO_3 (1.12 g) followed by the careful dilution with water to a total volume of 8.33 mL. Jones' reagent (1.1 mL) was added dropwise to a stirred solution of CHB (0.106 g, 1.0 mmol) in acetone (4 mL) in an ice−water bath. After complete addition of Jones' reagent, the ice−water bath was removed, and the mixture was stirred at room temperature for 1 h. Methanol (0.4 mL) was then added to remove excess of Jones' reagent. Approximately 3 mL of water was added, and the mixture was extracted three times with diethyl ether. The organic phase was washed several times with saturated NaCl solution until it became colorless. After it was dried with anhydrous $MgSO_4$, the solvent was removed. A pale yellow (74 mg, 71%) liquid was obtained. The obtained CBO, whose purity was ~90% as determined by GC-MS, was directly used in the experiments without further purification. Pure CBO (>98%), which was obtained by purification on a silica gel column with dichloromethane as the eluent, was used as a reference for HPLC analysis.

Reactions of CBO with GSH and Identification of Three CBO−GSH Conjugates. These reactions were carried out to prepare the standards for identification and quantification of CBO−GSH conjugates produced in the enzymatic reactions. All reactions were run in 50 mM, pH 7.4, Tris-HCl buffer at 37 °C. To prepare the di-GSH conjugate **5**, CBO (10 mg, 0.096 mmol) in Tris buffer (0.5 mL) was added to a solution of GSH (88.2 mg, 0.287 mmol, CBO:GSH = 1:3) in 0.5 mL of buffer. The mixture was incubated in a shaking water bath for 30 min, and then, 40 μL of 35% perchloric acid was added to terminate the reaction. Compound **5** (13 mg) was obtained through fraction collection on preparative HPLC and then lyophilization. To prepare the mono-GSH conjugates **3** and **4**, a CBO:GSH molar ratio of 2:1 was used. CBO (8.5 mg, 0.081 mmol) in 0.3 mL of buffer was added to a solution of GSH (10.7 mg, 0.0348 mmol) in 0.3 mL of buffer. After the reaction was allowed to proceed at 37 °C for 10 min, the reaction mixture was subjected to HPLC separation as described above. Compounds **3** (3.2 mg) and **4** (0.5 mg) were obtained through fraction collection and lyophilization.

Spectroscopic data of **5**: ESI-MS: *m/z* 683.5. ^{1}H NMR (400 MHz, D_2O): δ 4.63−4.55 (m, 2H, H-6 and H-16), 3.80−3.75 (m, 6H, H-8, H-13, H-18, and H-23), 3.14−3.00 (m, 3H, H-5 and H-4a), 2.92−2.81 (m, 3H, H-15 and H-4b), 2.57−2.51 (m, 4H, H-11 and H-21), 2.24−2.12 (m, 8H, H-1, H-3, H-12, and H-22).

Conversion of CHB to CBO by ADH. The enzymatic reaction was carried out at in vitro physiological conditions (pH 7.4, 37 °C). The reaction mixtures were prepared by adding ADH, NAD, and GSH solutions (100 μL each, made in 50 mM, pH 7.4, Tris-HCl buffer) to 100 μL of Tris-HCl in glass test tubes (100 mm × 16 mm). The test tubes were then placed in a shaking water bath at 37 °C for 3 min. Then, CHB solution in Tris-HCl buffer (100 μL) was added, and the reactions were allowed to proceed for 5−30 min. The total volumes of the solutions were 500 μL, and the final concentrations of the substances were as follows: ADH, 0.25 mg/tube; NAD, 1.5 mM; GSH, 1 or 5 mM; and CHB, 5 or 20 mM. Control incubations without ADH, GSH, or CHB were also carried out. All incubations were terminated by adding 20 μL of 35% perchloric acid to each test tube. The reaction mixtures were cooled down on ice and then centrifuged at 3000 rpm for 10 min to precipitate the protein. The supernatants were analyzed by HPLC with UV detection at 220 nm.

To determine the kinetic constants of the enzymatic reaction, eight CHB concentrations (0.25, 0.5, 1, 2.5, 5, 10, 15, and 20 mM) were used. The mixtures were incubated for 15 min with the GSH concentrations at 5 mM. Under these experimental conditions, only **5** was consistently detected, whereas **3** and **4** were trace products. Therefore, only the amounts of **5** were determined and used in the calculations.

Conversion of CHB to CBO by Rat Liver Cytosol. Because rat liver cytosol contains ADH, which has similar functions to horse liver ADH, CHB (5 mM) incubations were also carried out with rat liver cytosol (0.25 mg protein) and 5 mM GSH at pH 7.4 and 37 °C for 15, 30, and 60 min. The total volumes of the solutions were 500 μL. The control experiments without CHB, GSH, or rat liver cytosol were also performed.

CBO-Induced Globin Cross-Linking Analyses by SDS-PAGE. To obtain evidence for the ability of CBO to form protein cross-links at physiological conditions, freshly isolated mouse erythrocytes were incubated with CBO. After the removal of the plasma fraction from the whole blood of male C57B1 mice, erythrocytes were washed three times with an equal volume of saline and centrifuged at 3000 rpm for 5 min in between washes and then were resuspended in an equal volume of phosphate-buffered saline (8.4 mM Na_2HPO_4, 1.6 mM KH_2PO_4, and 154 mM NaCl, pH 7.4). Incubation with CBO was carried out in a Dubnoff metabolic shaking water bath at 37 °C for 1 or 24 h as previously described.[38] The final concentrations of CBO were 0.2, 0.5, 1, 2, 5, and 10 mM. At the end of the incubations, erythrocytes were lysed with equal volumes of cold doubly deionized water (ddH_2O), and globin was isolated using acidified acetone as described previously.[39] Globin (2.5 μg) dissolved in 2.5 μL of ddH_2O was added to the treatment buffer (500 mM, pH 7.4, Tris-HCl, 10% glycerol, 10% SDS, 0.01 g/mL bromophenol blue, and 10% 2-mercaptoethanol). After the addition of DTT (200 mM final concentration) to reduce dimer formation between globin chains due to disulfide bonds, the samples were kept at room temperature for 30 min before being loaded onto a 12.5% Tris-HCl Criterion Precast gel and run at 200 V for 75 min. Gels were silver stained for visualization of bands.

RESULTS

Reactions of CBO with GSH under in Vitro Physiological Conditions and Identification of Two CBO−Mono-GSH Conjugates and One CBO−Di-GSH Conjugate. The reaction of CBO with GSH under in vitro physiological conditions (pH 7.4, 37 °C) was performed first with an excess of GSH (the molar ratio of CBO:GSH was 1:3). After 30 min, the HPLC chromatogram of the reaction mixture indicated that CBO had been completely consumed, and only one product peak at 7.1 min could be observed. The protonated molecular ion peak of the product appeared at *m/z* 683.5 (Figure 1A), which was consistent with a structure

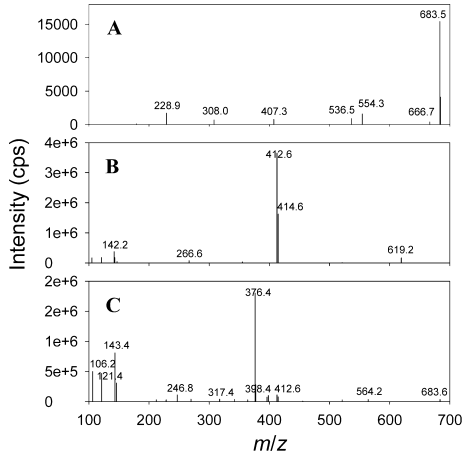
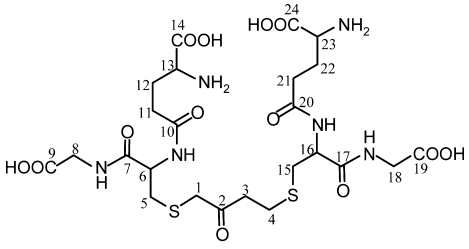


Figure 1. ESI mass spectra of the three CBO−GSH conjugates. (A) The CBO−di-GSH conjugate 1,4-*bis*(*S*-glutathionyl)butan-2-one (**5**). (B) The CBO−mono-GSH conjugate 1-chloro-4-(*S*-glutathionyl)-butan-2-one (**3**). (C) The CBO−mono-GSH conjugate 1-(*S*-glutathionyl)-3-buten-2-one (**4**).

that two molecules of GSH were added to one molecule of CBO. The ^{1}H NMR spectrum in D_2O indicated the presence of 26 protons. Comparison of the NMR data with published NMR spectroscopic data of GSH and **3**[40] showed that the spectrum was consistent with the expected structure 1,4-*bis*(*S*-glutathionyl)butan-2-one (**5**). Therefore, **5** was the product with the both ends (i.e., C-1 and C-4) of CBO reacting with GSH; namely, it was the di-GSH conjugate of CBO (Scheme 2).

Scheme 2. Structure of the Di-GSH Conjugate of CBO (5)



5

When the reaction of CBO with GSH was performed with an excess of CBO (the molar ratio of CBO:GSH was 2:1) at pH 7.4 and 37 °C for 10 min, two HPLC peaks were observed at 9.4 and 14.7 min in addition to the peaks for **5** and unreacted GSH and CBO (their retention times were 7.1, 4.9, and 19.6 min, respectively). The ESI-MS of the compound eluted at 14.7 min showed a protonated molecular ion peak at *m/z* 412.6 with an isotope peak at *m/z* 414.6 (~1/3 of the intensity of the peak at *m/z* 412.6) (Figure 1B), indicating that the compound contains a chlorine atom. The molecular weight was consistent with a structure that GSH was added to C-4 of CBO through Michael addition. The protonated molecular ion peak of the compound eluted at 9.4 min appeared at *m/z* 376.4 (Figure 1C), which was consistent with a structure that chlorine atom in CBO was replaced by GSH. In addition, the isolated

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compounds **3** and **4** both were readily converted to **5** upon further incubation with GSH, providing evidence for these compounds being the mono-GSH conjugates of CBO through reactions on C-4 and C-1 of CBO, respectively. Thus, the two products were identified as 1-chloro-4-(*S*-glutathionyl)butan-2-one (**3**) and 1-(*S*-glutathionyl)-3-buten-2-one (**4**), respectively. The reaction of CBO with GSH has been reported by Munter et al. to yield two products: **5**, as identified solely by mass spectrometry, and **3**, which was identified by both NMR and mass spectrometry.[40]

Conversion of CHB to CBO by ADH. To examine if CHB could be oxidized by ADH, CHB (5 mM) was incubated with purified horse liver ADH at pH 7.4 and 37 °C. Because CBO is a bifunctional alkylating agent, 5 mM GSH was included in the incubations to serve as a trapping agent. To confirm that the formation of CBO was attributed to the presence of ADH, a control experiment without ADH was carried out. The three CBO−GSH conjugates described above were used as the standards for identification and quantification of the enzymatic products.

The results showed that three products, including one major and two minor ones, were formed. The major product was identified as **5**, and the two minor ones were **3** and **4**. The amount of **5** increased over time, and those of **3** and **4** changed little with time (Figure 2A). In addition, a small amount of

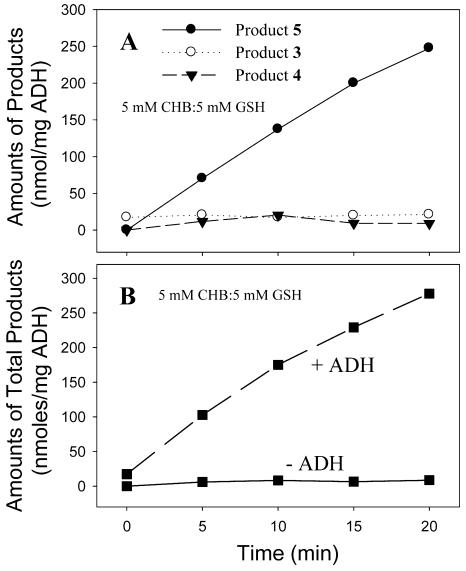

Figure 2. (A) Time-dependent formation of the three CBO−GSH conjugates **3**−**5** when CHB was incubated with horse liver ADH under in vitro physiological conditions (pH 7.4, 37 °C). (B) Representative graph showing the non-ADH-dependent formation of the CBO−GSH conjugates (the total amounts) as compared to that formed in the presence of ADH. All incubations contained 5 mM CHB, 1.5 mM NAD, and 5 mM GSH. The ADH-containing incubations had 0.25 mg of ADH.

CBO was also detected (data not shown). When ADH was absent, only a trace amount of **3** was formed, probably due to nonenzymatic oxidation of CHB by oxygen (Figure 2B). In the control experiments without GSH, a significant increase in the amount of CBO over time was observed (data not shown). A comparison of the CBO amounts in the absence of GSH with those in the presence of GSH revealed that ∼90% of the CBO detected in the incubation without GSH was consumed when GSH was included in the incubation. When higher CHB (20 mM) and lower GSH concentrations (1 mM) were used, ∼70% of the CBO detected in the absence of GSH was consumed when GSH was included. Collectively, the results clearly indicated that CHB was converted to CBO by purified ADH, and the formed CBO in turn reacted with GSH to yield the three CBO−GSH conjugates **3**−**5**.

Dependence of the Amounts and Ratios of the CBO−GSH Conjugates Formed on the CHB and GSH Concentrations. To examine how altering the CHB and GSH concentrations could affect the amounts and ratios of the CBO−GSH conjugates produced, two experiments with different CHB and GSH concentrations (20:1 mM and 5:5 mM, respectively) were performed.

The total amounts of the three conjugates increased over time in both experiments (Figure 3A), indicating that CBO was

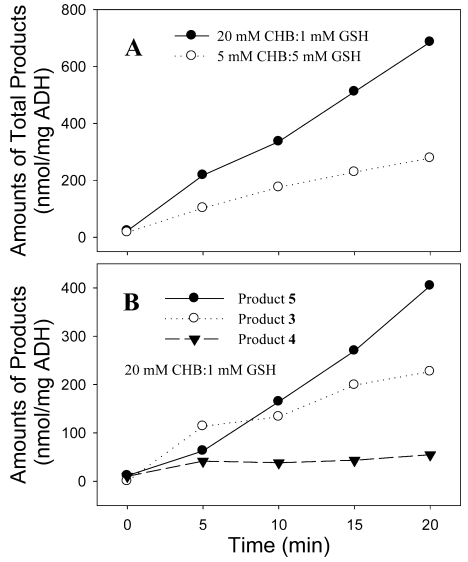

Figure 3. Time-dependent formation of the CBO−GSH conjugates when CHB was incubated with horse liver ADH. (A) Total CBO−GSH conjugates formed at 20 mM CHB and 1 mM GSH or at 5 mM CHB and 5 mM GSH. (B) Formation of the CBO−GSH conjugates **3**−**5** at 20 mM CHB and 1 mM GSH. All incubations were carried out using 0.25 mg of ADH and 1.5 mM NAD. Values were corrected for any non-ADH-dependent formation at all time points examined.

continuously produced. The total amounts of the conjugates formed at 20 mM CHB were approximately 2−2.5-fold larger than those observed at 5 mM CHB (Figure 3A).

The ratios of the three conjugates were dependent on the concentrations of CHB and GSH. When the CHB concentration was lower (5 mM) and the GSH concentration was higher (5 mM), the di-GSH conjugate **5** was the overwhelmingly predominant product (Figure 2A), apparently due to a large excess of GSH over CBO formed. However, with higher CHB concentration (20 mM) and lower GSH concentration (1 mM), although generally **5** was still the predominant product, the yields of the two mono-GSH conjugates **3** and **4** greatly increased (Figure 3B). In particular, the amount of **3**, which increased by up to 11 times in comparison with that in the experiment with 5 mM GSH, was greater than that of **5** detected at the 5 min time point (Figure 3B).

Determination of Kinetic Constants of the Enzymatic Reaction. The conversion of CHB to CBO by purified ADH

followed the Michaelis–Menten kinetics (Figure 4). The K_m and k_{cat} were determined as 3.5 mM and 0.033 s^{-1}, respectively. In the experiments, the GSH concentration was 5 mM, and the incubation time was 15 min.

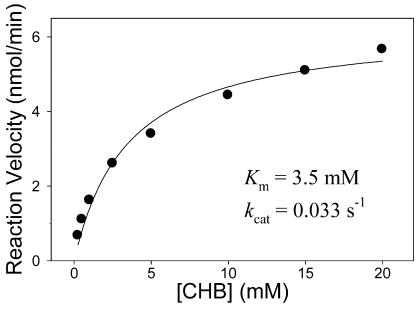

Figure 4. ADH-dependent conversion of CHB to CBO, as measured by the CBO–di-GSH conjugate **5**, followed Michaelis–Menten kinetics. The K_m and k_{cat} were determined to be 3.5 mM and 0.033 s^{-1}, respectively. The GSH concentrations were 5 mM, and the incubation time was 15 min.

Biotransformation of CHB to CBO by Rat Liver Cytosol. Because liver cells contain abundant ADH, it was expected that CHB could be converted to CBO by liver cytosol. To confirm it, a mixture of 5 mM CHB and 5 mM GSH was incubated with rat liver cytosol at 37 °C for 15, 30, and 60 min. The results showed that all three CBO–GSH conjugates were formed over time (Figure 5). Interestingly, the CBO–mono-

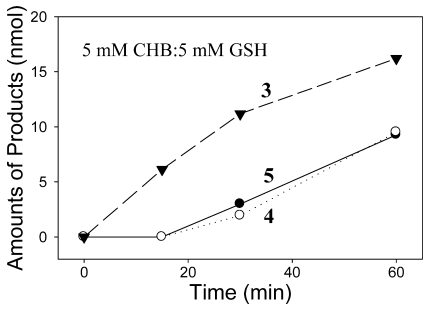

Figure 5. Time-dependent formation of the CBO–GSH conjugates **3**–**5** after CHB (5 mM) was incubated with rat liver cytosol (0.25 mg protein). The reaction mixtures also contained 5 mM GSH and were incubated at pH 7.4 and 37 °C.

GSH conjugate **3** was the predominant product in the experiment, whereas the CBO–mono-GSH conjugate **4** and the CBO–di-GSH conjugate **5** were minor products, consistent with the increased formation of **3** at the early time points when purified ADH was incubated with 20 mM CHB and 1 mM GSH (Figure 3B).

CBO-Induced Cross-Linking of Hemoglobin. The reaction of CBO with GSH produced predominantly the di-GSH conjugate **5**, indicating that CBO was a bifunctional alkylating agent. Thus, CBO could probably induce cross-linking of proteins. To obtain evidence for the protein cross-linking ability of CBO at physiological conditions, mouse erythrocytes were incubated with different concentrations of CBO (0.2, 0.5, 1, 2, 5, and 10 mM) for 1 or 24 h, and then, globin was isolated and subjected to SDS-PAGE and silver staining. Indeed, CBO-induced globin dimer bands were observed. When erythrocytes were incubated with 10 mM CBO for 24 h, globin isolated exhibited much more visible darker dimer bands (Figure 6, lanes 2, 4, and 6) in comparison

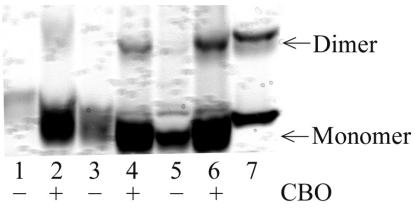

Figure 6. SDS-PAGE and silver staining of globin samples isolated after erythrocytes were incubated with (lanes 2, 4, and 6) and without CBO (10 mM) (lanes 1, 3, and 5). The incubations were carried out at 37 °C for 24 h. Lanes 1 and 2 contained 5 μg of protein, lanes 3 and 4 contained 10 μg of protein, and lanes 5 and 6 contained 20 μg of protein. Lane 7 contained the molecular weight marker. The gel image represents the results obtained from a typical experiment.

with the corresponding control samples incubated with buffer only (Figure 6, lanes 1, 3, and 5). Concentration-dependent increases in dimer band intensity were also observed when the CBO concentration was increased from 0.2 to 10 mM and the incubation time was 1 h (data not shown). Collectively, the results indicated that CBO was able to cause globin cross-linking in erythrocytes.

DISCUSSION

CHB, which was formed when BD was incubated with MPO in the presence of H_2O_2 and chloride ion,[25] is a structural analogue of BDD. Similar to BDD, which was previously shown to be oxidized to HMVK by purified horse liver ADH and cytosolic ADH from mouse, rat, and human liver,[29] and also by human cytochromes P450s,[30] the results presented in the present study provide clear evidence for CHB oxidation by isolated horse liver ADH and cytosolic ADH from rat liver to yield CBO, which, in turn, was a structural analogue of HMVK.

Although CBO was expected to have reactivity toward biological nucleophiles similar to that of HMVK due to their similar structures, there are significant differences in reactivity between the two compounds. Probably the principal difference is that only one end of HMVK has reactivity, whereas both ends of CBO are reactive. Thus, unlike HMVK, CBO is a bifunctional alkylating agent, which was confirmed by the fact that the di-GSH conjugate **5** was readily formed in the CBO–GSH reactions. The bifunctional alkylating ability of CBO was further demonstrated by the formation of dimer bands of globin when CBO was incubated with mouse erythrocytes.

Evidence for BDD oxidation to HMVK in mice and rats in vivo[41] was obtained by the detection of the urinary metabolite 4-(*N*-acetyl-L-cystein-*S*-yl)-1,2-dihydroxybutane (the mercapturic acid of HMVK–GSH conjugate)[32] and detection of HMVK–globin adducts in rats and mice given BDD.[33] As a reactive Michael acceptor, HMVK exhibited high reactivity toward nucleophilic amino acids,[42] hemoglobin,[43] and nucleosides and DNA.[44] Thus, it is expected that CHB can also be biotransformed to CBO in vivo, which can react with a variety of biomacromolecules. Because of its high reactivity and cross-linking ability, CBO may be highly mutagenic and could play a significant role in the mutagenicity/carcinogenicity of BD.

Previous studies provide evidence for the presence of both a high-affinity pyrazole-insensitive ADH and a low-affinity pyrazole-sensitive ADH in human blood and bone marrow cells.[45,46] Human bone marrow has also been reported to

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express mRNA of cytochrome P450 1A1, 2A6/7, 2D6, 2E1, and 3A4.[47] Cytochrome P450 2E1 was also detected by Western blotting in human $CD34^+$ bone marrow stem cells.[48] In addition, human blood monocytes and macrophages contain multiple P450s (P450 1A1, 1A2, 1B1, 2B6/7, 2E1, 3A3/4, 3A7, and 4B1) as determined by RT-PCR.[49] Thus, it is reasonable to speculate that CHB can undergo ADH- and cytochrome P450s-mediated conversion to CBO in human bone marrow and immune cells.

Chlorohydrins, such as ethylene chlorohydrin, propylene chlorohydrin, and 3-chloro-1,2-propanediol, are considered potential toxicants and carcinogens.[50−56] In this regard, exposure to chlorohydrins have been associated with leukemia and pancreatic cancers[54] and lymphatic and hematopoietic cancers.[55,56] Thus, mutagenicity and toxicity of CHB warrant further investigations.

Although DEB is widely considered the most likely metabolite responsible for mutagenicity/carcinogenicity of BD, its blood concentration is thought to be extremely low in humans exposed to BD on the basis of data of the DEB-specific hemoglobin adducts,[57,58] probably due to the presence of high levels epoxide hydrolase activities in humans.[2] After analyzing published data using a cancer risk model on the basis of the concentration of the epoxide metabolites in the blood over time and their mutagenic potency, Fred et al. suggested that different epoxide metabolites were predominating cancer-initiating agents in the cancer tests with BD, DEB in the mouse, and monoepoxides (EB and EBD) in the rat.[24] Because DEB concentrations in humans appear even lower than corresponding concentrations in rats,[57,58] it seems reasonable to speculate that DEB is not the predominant cancer-initiating metabolite in humans. The predominant metabolites responsible for mutagenicity/carcinogenicity of BD in humans could thus be monoepoxides and/or CHB/CBO.

CHB is a BD metabolite likely to be exclusively generated in immune and bone marrow cells because its formation requires the presence of MPO and high concentrations of H_2O_2 and chloride ion. Immune cells, for example, lymphocytes and neutrophil granulocytes, are also formed in the bone marrow. These findings and the epidemiological studies indicating a causal association between exposure to BD and excess lymphohematopoietic cancers in workers occupationally exposed to BD[1] suggest that CHB and/or CBO may play a role in lymphohematopoietic cancers induced by BD in humans. Thus, studies investigating CHB and CBO formation in vivo are warranted and may clarify the potential roles of CHB and CBO in BD toxicity and carcinogenicity.

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ABBREVIATIONS

ADH, alcohol dehydrogenase; BD, 1,3-butadiene; BDD, 3-butene-1,2-diol; CBO, 1-chloro-3-buten-2-one; CHB, 1-chloro-2-hydroxy-3-butene; ddH_2O, doubly deionized water; DEB, 1,2,3,4-diepoxybutane; EB, 3,4-epoxy-1-butene; EBD, 3,4-epoxy-1,2-butanediol; ESI, electrospray ionization; GSH, glutathione; HMVK, hydroxymethylvinyl ketone; MPO, myeloperoxidase; NAD, nicotinamide adenine dinucleotide; TFA, trifluoroacetic acid

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