Metabolic Activation of 1,2-Dibromo-3-chloropropane: Evidence for the Formation of Reactive Episulfonium Ion Intermediates†

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ABSTRACT: The nematocide and soil fumigant 1,2-dibromo-3-chloropropane (DBCP) is a carcinogen and a mutagen and displays target-organ toxicity to the testes and the kidney. It has been proposed that both cytochrome P-450 mediated activation and glutathione (GSH) conjugation pathways are operative in DNA damage and organotropy induced by DBCP. To determine the chemical mechanisms involved in the bioactivation of DBCP and to assess a role for an episulfonium ion intermediate, the mechanism of formation of GSH conjugate metabolites of DBCP was investigated. Five biliary GSH conjugates of DBCP were isolated from rats and identified by fast atom bombardment tandem mass spectrometry: *S*-(2,3-dihydroxypropyl)glutathione (I), *S*-(2-hydroxypropyl)glutathione (IIA), *S*-(3-chloro-2-hydroxypropyl)glutathione (III), 1,3-di(*S*-glutathionyl)propan-2-ol (IV), and 1-(glycyl-*S*-cysteinyl)-3-(*S*-glutathionyl)propan-2-ol (V). The mechanisms of conjugate formation were addressed by assessing deuterium retention in conjugates derived from [1,1,2,3,3-$^2H_5$]DBCP (D_5-DBCP). GSH conjugates I, III, IV, and V displayed quantitative retention of deuterium, an observation consistent with the formation of an episulfonium ion intermediate. GSH conjugate IIA, however, retained three atoms of deuterium, thus invoking a P-450 mechanism in its genesis. The involvement of glutathione transferase (GST) and sequential episulfonium ion intermediates in the formation of metabolites I, III, and IV was demonstrated in vitro. Upon incubation of DBCP with GST, metabolites I, III, and IV were identified by tandem mass spectrometry and were found to arise with quantitative retention of deuterium when D_5-DBCP was employed as a substrate. An additional GSH conjugate, 1,2,3-tri(*S*-glutathionyl)propane (VI), was observed as the major metabolite in incubations of GST with DBCP. When the incubations of DBCP with GST were performed in $H_2{}^{18}O$, metabolite I incorporated two atoms of ^{18}O, and metabolites III and IV incorporated one atom of ^{18}O. The ability of GST to catalyze the formation of the four GSH conjugates observed in vivo, with quantitative retention of deuterium and incorporation of ^{18}O from $H_2{}^{18}O$, may be rationalized by a mechanism invoking the initial formation of *S*-(2-bromo-3-chloropropyl)glutathione. Rearrangement of this unstable conjugate via several reactive episulfonium ions, with either hydrolysis by water or alkylation of GSH at various stages, would account for the pattern of metabolites and their status of isotopic enrichment observed under various incubation conditions. The compelling chemical evidence presented here for the formation of episulfonium ion intermediates is in accord with mechanistic studies conducted on DBCP-induced DNA damage and toxicity in extrahepatic tissues, in which indices of toxicity were found to be insensitive to deuterium substitution.

The haloalkane nematocide 1,2-dibromo-3-chloropropane (DBCP)[1] was used extensively as a soil fumigant prior to the observation of testicular atrophy, oligiospermia, and infertility in male pesticide formulators exposed to DBCP (Whorton et al., 1977). In experimental animals the acute toxicity of DBCP is characterized by necrosis of the renal proximal tubules, testicular atropy accompanied by oligiospermia (Kluwe, 1981a,b), and occasional liver damage. Chronic administration of DBCP to animals has resulted in tumors of the nasal turbinates, forestomach, and liver (National Cancer Institute, 1980; Whorton & Foliart, 1983). Initial studies on the mechanism of DBCP bioactivation have proposed activation via the intermediacy of an electrophilic epihalohydrin (epichlorohydrin or epibromohydrin; Jones et al., 1979) which was proposed to bind irreversibly to tissue macromolecules (Kato et al., 1980). This suggestion was supported by the observation that 3-chloro-1,2-propane oxide (epichlorohydrin) and 3-chloropropanediol (α-chlorohydrin) produce testicular lesions similar to those observed after DBCP exposure (Kluwe et al., 1983). More recently, however, a role for epichlorohydrin in the in vivo activation of DBCP has been refuted (Gingell et al., 1986). Moreover, studies with inducers or inhibitors of cytochrome P-450 have failed to implicate an oxidative mechanism in the extrahepatic toxicity of DBCP (Kluwe, 1983).

DBCP is genotoxic in a number of short-term mutagenesis assays (Blum & Ames, 1977; Whorton & Foliart, 1983). Mechanistic studies conducted in vitro with selectively deuterated analogues of DBCP have demonstrated that DBCP

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[1] Abbreviations: CID, collision-induced dissociation; DBCP, 1,2-dibromo-3-chloropropane; D_5-DBCP, [1,1,2,3,3-$^2H_5$]-1,2-dibromo-3-chloropropane; FAB/MS, fast atom bombardment mass spectrometry; GSH, glutathione; GST, glutathione transferase; HPLC, high-performance liquid chromatography; MS–MS, tandem mass spectrometry; I, *S*-(2,3-dihydroxypropyl)glutathione; IIA, *S*-(2-hydroxypropyl)glutathione; IIB, *S*-(3-hydroxypropyl)glutathione; III, *S*-(3-chloro-2-hydroxypropyl)glutathione; IV, 1,3-di(*S*-glutathionyl)propan-2-ol; V, 1-(glycyl-*S*-cysteinyl)-3-(*S*-glutathionyl)propan-2-ol; VI, 1,2,3-tri(*S*-glutathionyl)propane.

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is oxidized by cytochrome P-450 to mutagenic metabolites, and a good correlation between the mutagenesis of DBCP and the metabolic generation of the potent, direct-acting mutagen 2-bromoacrolein has been established (Omichinski et al., 1988a). It has been suggested recently that 2-bromoacrolein or 1-bromo-3-chloroacetone may be involved in the testicular toxicity or nephrotoxicity of DBCP (Dohn et al., 1988). Administration of deuterated analogues of DBCP to rats, however, failed to alleviate the changes in biochemical or morphological indices of kidney damage induced by DBCP (Omichinski et al., 1987). In a similar manner no significant difference in the potency of DBCP or D_5-DBCP to cause testicular cell DNA damage or necrosis was observed either in vivo (Soderlund et al., 1988) or in vitro (Omichinski et al., 1988b). These observations suggest that oxidative metabolism is important in the mutagenesis of DBCP but implicate a bioactivation pathway independent of cytochrome P-450 in the expression of DBCP-induced extrahepatic toxicities.

DBCP is structurally related to the soil fumigant and gasoline additive 1,2-dibromoethane, a compound known to be bioactivated by a GSH-dependent mechanism (Koga et al., 1986; van Bladeren et al., 1980; Working et al., 1986). DBCP causes depletion of GSH in the liver, kidneys, testes, and forestomach of rats (Kluwe et al., 1981), and cytosolic fractions from these organs catalyze the consumption of GSH when coincubated with DBCP (Kluwe et al., 1981; Mcfarland et al., 1984). Toxicity studies have inferred a role for GSH-dependent metabolism in DBCP-related extrahepatic toxicities (Omichinski et al., 1988b). The nature of the reactive intermediate(s), however, and their mechanism of biogenesis have not been well-defined chemically, unlike other vicinally dihalogenated compounds such as 1,2-dibromoethane (Peterson et al., 1988; Inskeep et al., 1986) and 1,2-dichloroethane (Webb et al., 1987). Therefore, identification of GSH conjugates of DBCP formed in vivo and strategies to elucidate their mechanism of formation represent important goals in understanding the metabolic and chemical basis for DBCP-induced target-organ toxicities.

The present investigation focuses on the identification of GSH conjugates of DBCP by tandem mass spectrometry and addresses their mechanism of formation by use of stable isotopes as mechanistic probes. In this paper we present compelling evidence for the intermediacy of reactive episulfonium ion metabolites in the bioactivation of DBCP.

Experimental Procedures

Chemicals. Epichlorohydrin, epibromohydrin, glycidol, propargyl alcohol, propylene oxide, 3-bromopropanol, benzyl chloroformate, and sodium borodeuteride (98 atom %) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Glutathione and rat liver glutathione transferase were obtained from Sigma Chemical Co. (St. Louis, MO). Deuterium oxide (D_2O; 99.8 atom %) was obtained from Cambridge Isotope Laboratories (Woburn, MA). Lithium aluminium deuteride (99 atom %) was purchased from Stohler Isotope Chemicals (Waltham, MA). Oxygen-18-enriched water (91.7 atom % $H_2{}^{18}O$) was obtained from Isotec Inc. (Miamisburg, OH). [U-^{14}C]DBCP (23.6 mCi mmol^{-1}; 97% radiochemically pure) was a gift from Shell Development Co.

Synthetic Procedures. High-field proton NMR (^{1}H NMR) spectra were obtained on a Varian VXR-300 spectrometer operating at 300 MHz, samples were dissolved in D_2O, and the chemical shifts are expressed in parts per million (δ) downfield from sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) as an internal standard. The residual $^1H^2HO$ signal at 4.7–4.8 ppm was attenuated by continuous irradiation with the decoupler. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

[1,1,2,3,3-$^2H_5$]DBCP (D_5-DBCP) was prepared as previously described (Omichinski & Nelson, 1988). Isotopic composition was determined by EIMS to be 16% $^2H_4$ and and 84% $^2H_5$ on the basis of the M − Br ions at m/z 155–165. The sample was 95% pure by GC analysis.

Synthesis of GSH Conjugates. The synthesis of four GSH conjugate metabolites of DBCP and a regioisomer of metabolite II was achieved by a general strategy in which GSH was treated with an appropriate alkylating agent in basic methanol. The conjugates were subsequently converted to their *N*-(benzyloxycarbonyl) derivatives, and analytically pure standards were obtained by reverse-phase HPLC purification.

S-(2,3-Dihydroxypropyl)glutathione (I). Glutathione (500 mg; 1.6 mmol) and sodium metal (73.6 mg; 3.2 mmol) were dissolved in dry methanol (25 mL). Glycidol (170.2 mg; 2.4 mmol) was added in 1 mL of dry methanol, and the reaction was stirred under an argon atmosphere at ambient temperature for 12 h. The methanol was removed in vacuo, and the residue was resuspended in 100 mM ammonium formate buffer (pH 9.0; 20 mL) and treated with benzyl chloroformate (1 mL) for 15 min at ambient temperature. Excess benzyl chloroformate was removed by extraction with dichloromethane (20 mL); the residual aqueous portion was lyophilized. An analytically pure sample of I as its *N*-(benzyloxycarbonyl) derivative was obtained by preparative HPLC as described for HPLC purification of GSH conjugates. ^{1}H NMR (D_2O): δ 7.5 (5 H, m; Ar-*H*); 5.13 (2 H, s; OCH_2Ph); 4.6 (1 H, m; Cys-α-CH); 4.2 (1 H, dd, J = 4 and 10 Hz; Glu-α-CH); 3.96 (2 H, s; Gly-α-CH); 3.8 (1 H, m; –C*H*OH–); 3.57 (2 H, m; –CH_2OH); 3.05 (1 H, dd, J = 14 and 4 Hz; Cys-β-CH); 2.88 (1 H, m; Cys-β-CH); 2.72 (1 H, m; *S*-CH_2–CHOH–); 2.62 (1 H, m; *S*-CH_2–CHOH–); 2.43 (2 H, t; J = 7 Hz, Glu-γ-CH); 2.23 (1 H, m; Glu-β-CH); 1.99 (1 H, m; Glu-β-CH). FAB/MS (m/z): 516 (MH$^+$).

S-(2-Hydroxypropyl)glutathione (IIA). Glutathione (500 mg; 1.6 mmol) was reacted with propylene oxide (132 mg; 2.4 mmol) in the presence of sodium metal (73.6 mg; 3.2 mmol) dissolved in dry methanol (25 mL). The product was converted to its *N*-(benzyloxycarbonyl) derivative and purified as described for I. ^{1}H NMR (D_2O): δ 7.4 (5 H, m; Ar-*H*); 5.1 (2 H, s; OCH_2Ph); 4.5 (1 H, m; Cys-α-CH); 4.15 (1 H, dd, J = 5 and 11 Hz; Glu-α-CH); 3.95 (2 H, s; Gly-α-CH); 3.9 (1 H, m; –C*H*OH–); 3.0 (1 H, dd, J = 14 and 5 Hz; Cys-β-CH); 2.8 (1 H, m; Cys-β-CH); 2.65 (1 H, m; *S*-CH_2–CHOH–); 2.65 (1 H, m; *S*-CH_2–CHOH–); 2.4 (2 H, t, J = 7 Hz; Glu-γ-CH); 2.2 (1 H, m; Glu-β-CH); 1.95 (1 H, m; Glu-β-CH); 1.15 (3 H, d, J = 6 Hz; –CH$_2$OHCH_3). FAB/MS (m/z): 500 (MH$^+$).

S-(3-Hydroxypropyl)glutathione (IIB). Glutathione (500 mg; 1.6 mmol) was reacted with 3-bromopropanol (390 mg; 3.2 mmol) in the presence of sodium metal (73.6 mg; 3.2 mmol) dissolved in dry methanol (25 mL). The product was converted to its *N*-(benzyloxycarbonyl) derivative and purified as described for I. ^{1}H NMR (D_2O): δ 7.5 (5 H, m; Ar-*H*); 5.2 (2 H, s OCH_2Ph); 4.65 (1 H, m; Cys-α-CH); 4.05 (1 H, dd, J = 4 and 9 Hz; Glu-α-CH); 3.85 (2 H, s; Gly-α-CH); 3.75 (2 H, m; –CH_2OH); 3.1 (1 H, dd, J = 14 and 5 Hz; Cys-β-CH); 2.9 (1 H, m; Cys-β-CH); 2.7 (2 H, m; *S*-CH_2–CH$_2$–); 2.5 (2 H, t, J = 7 Hz; Glu-γ-CH); 2.2 (1 H, m; Glu-β-CH); 1.9 (2 H, m; *S*-CH$_2$–CH_2–CHOH–); 1.85 (1 H, m; Glu-β-CH). FAB/MS (m/z): 500 (MH$^+$).

S-(3-Chloro-2-hydroxypropyl)glutathione (III). Glutathione (500 mg; 1.6 mmol) was reacted with epichlorohydrin

(211.6 mg; 2.4 mmol) in the presence of sodium metal (73.6 mg; 3.2 mmol) dissolved in dry methanol (25 mL). The product was converted to its *N*-(benzyloxycarbonyl) derivative and purified as described for I. ^{1}H NMR (D_2O): δ 7.45 (5 H, m; Ar-*H*); 5.12 (2 H, s; OCH_2Ph); 4.55 (1 H, m; Cys-α-CH); 4.14 (1 H, dd, J = 4 and 10 Hz; Glu-α-CH); 3.96 (1 H, m; -C*H*OH-); 3.9 (2 H, s; Gly-α-CH); 3.65 (2 H, m; $-CH_2Cl$); 3.05 (1 H, dd, J = 14 and 4 Hz; Cys-β-CH); 2.85 (1 H, m; Cys-β-CH); 2.7 (2 H, m; S-CH_2-CHOH-); 2.45 (2 H, t, J = 7 Hz; Glu-γ-CH); 2.20 (1 H, m; Glu-β-CH); 1.95 (1 H, m; Glu-β-CH). FAB/MS (m/z): 534 (100%) and 536 (42%) (MH^+).

1,3-Di(S-glutathionyl)propan-2-ol (IV). Glutathione (1 g; 3.2 mmol) was reacted with epibromohydrin (208.5 mg; 1.6 mmol) in the presence of sodium metal (73.6 mg; 3.2 mmol) dissolved in dry methanol (25 mL). The product was converted to its *N,N*-bis(benzyloxycarbonyl) derivative, and an analytically pure sample of IV was obtained by preparative HPLC as described for HPLC purification of GSH conjugates. ^{1}H NMR (D_2O): δ 7.45 (10 H, m; Ar-*H*); 5.15 (4 H, s; OCH_2Ph); 4.55 (2 H, m; Cys-α-CH); 4.14 (2 H, m; Glu-α-CH); 3.9 (4 H, s; Gly-α-CH_2); 3.85 (1 H, m; -C*H*OH-); 3.05 (2 H, br d, J = 14 Hz; Cys-β-CH); 2.85 (2 H, m; Cys-β-CH); 2.75 (2 H, m; S-C*H*-CHOH-); 2.65 (2 H, m; S-C*H*-CHOH-); 2.45 (4 H, m; Glu-γ-CH); 2.20 (2 H, m; Glu-β-CH); 1.98 (2 H, m; Glu-β-CH). FAB/MS (m/z): 939 (MH^+).

HPLC Purification of Glutathione Conjugates. HPLC purification of *N*-(benzyloxycarbonyl)glutathione conjugates of DBCP or D_5-DBCP isolated from the bile of rats was performed on a system equipped with two Waters M6000A pumps, a Waters 440 UV detector (λ = 254 nm), and a Waters 680 HPLC gradient controller (Waters Associates, Milford, MA.) Separation of GSH conjugates was achieved on a reverse-phase Altex 5-μm Ultrasphere ODS (25 cm × 10 mm i.d., Rainin Instruments, Berkley, CA). The elution mobile phase consisted of a linear 30-min gradient from 30% to 95% methanol in water, with 1% acetic acid throughout, followed by isocratic elution at 95% methanol for an additional 10 min. The flow rate was held at 3 mL min^{-1}, and fractions of the column effluent were collected at 30-s intervals with a Pharmacia Frac-100 fraction collector (Pharmacia, Pitscaway, NJ). Aliquots (500 μL) of the HPLC fractions were added to Readysafe scintillation cocktail (5 mL), and the retention times of biliary metabolites of [U-^{14}C]DBCP were established by scintillation counting in a Packard Tricarb 2000CA liquid scintillation counter (Parkard Instrument Co., Downers Grove, IL); quench correction was performed in the external standardization mode, and the recovery of radioactivity in the HPLC eluent was greater than 95% of that applied to the column. The HPLC retention times of reference standards for glutathione conjugate metabolites of DBCP were established by monitoring the HPLC column effluent at λ = 254 nm following an injection of 50 μg of each authentic standard.

Mass Spectrometry. Fast atom bombardment mass spectra (FAB/MS) were recorded on a VG 70-SEQ hybrid tandem mass spectrometer, of *EBqQ* geometry (VG Analytical Ltd., Manchester, U.K.), equipped with an Ion Tech saddle-field fast atom gun and a VG 11/250 data system. Samples (1–5 μg) were dissolved in a glycerol matrix, containing HCl, on an FAB target. Ionization was achieved following bombardment with a primary beam of xenon (8 keV). Spectra were recorded via the data system at an accelerating voltage of 8 kV and a nominal mass resolution of $M/\Delta M$ = 2000 (10% valley). Daughter ion spectra (MS-MS) of the MH^+ ions of candidate metabolites of DBCP were obtained by collision-induced dissociation (CID) in the first (rf only) quadrupole, at collision energies of 20–40 eV (laboratory frame of reference). Argon was employed as a collision gas at a pressure of 2×10^{-6} Torr (in quadrupole analyzer housing) to fragment simple monoglutathione-containing conjugates. However, for polyglutathione conjugates, collision cell pressures of 7×10^{-6} Torr were required to afford structurally informative CID spectra. The parent MH^+ ions of the analytes were selected by adjustment of the magnetic field strength of the first mass analyzer (B), and daughter ion spectra were recorded by scanning the quadrupole mass analyzer (Q) from m/z 50 to m/z 1200 over a period of 10 s. The spectra were recorded in the MCA (multichannel analysis) mode; five to ten scans were summated, centroids were assigned, and the peaks were subsequently mass measured by the data system.

Animals and Treatment Procedures. Male Spague–Dawley rats (200–225 g; Charles River Laboratories Inc., Wilmington, MA) were maintained at 23–27 °C on a 12-h light/dark cycle. Tap water and laboratory chow (Ralston Purina Co., St. Louis, MO) were provided ad libitum. Rats were anesthetized with sodium hexobarbital (75 mg kg^{-1}), a polyethylene cannula (PE-10) was inserted into the bile duct, and bile flow was exteriorized via a midline incision. The rats were allowed to recover for 6 h with free access to food and water, at which time bile flow was stabilized at 1.0–1.2 mL h^{-1}. DBCP (80 mg kg^{-1}) or the perdeuterio analogue was administered with a radiotracer of [U-^{14}C]DBCP (1000 dpm nmol^{-1}) in 300 μL of DMSO, and the bile was collected for a further 6-h period. The excretion of [U-^{14}C]DBCP metabolites was monitored by liquid scintillation counting of bile aliquots (20 μL).

Isolation and Identification of Biliary Glutathione Conjugates of DBCP. Bile samples obtained from three rats administered DBCP were combined, diluted with 100 mM carbonate buffer (pH 9; 10 mL), and treated with 500 μL of benzyl chloroformate. Excess benzyl chloroformate was removed by extraction with dichloromethane (10 mL); the residual aqueous portion was acidified (to pH <2) and centrifuged (500g × 5 min) to remove any precipitate. The aqueous supernatant containing the derivatized GSH conjugates was applied to a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA), which had been prewashed with methanol (10 mL) and water (20 mL). The cartridge was rinsed with water (5 mL), and the benzyloxycarbonyl derivatives were eluted with methanol (5 mL) and evaporated to dryness under a stream of nitrogen. The residue was resuspended in the HPLC mobile phase, and aliquots (50 μL) were subjected to analysis by reverse-phase HPLC. The HPLC fractions comprising the five major radioactive peaks were identified by liquid scintillation counting, and the metabolites were concentrated on individual C_{18} Sep-Pak cartridges, as described above. The partially purified conjugates were subsequently treated with anhydrous methanolic HCl at ambient temperature for 2 h; the increased lipophilicity and increased retention time upon derivatization facilitated further purification of the conjugates as their *N*-(benzyloxycarbonyl) dimethyl ester derivatives. The radioactive fractions were taken for mass spectrometric analysis. Deuterium retention in the GSH conjugates isolated from the bile of rats administered D_5-DBCP was estimated by mass spectrometry from the average of five scans recorded across the molecular ion envelope of the appropriate analyte.

In Vitro Metabolism of DBCP by Rat Liver Glutathione Transferase. DBCP and D_5-DBCP (in an equimolar ratio) and a radiotracer of [U-^{14}C]DBCP (1 mM; 500 dpm nmol^{-1})

Table I: HPLC Retention Times of Derivatized GSH Conjugates of DBCP Excreted in the Bile of Rats

metabolite	HPLC retention time of derivatized GSH conjugates (min): *N*-(benzyloxycarbonyl) derivative	HPLC retention time of derivatized GSH conjugates (min): *N*-(benzyloxycarbonyl) dimethyl ester derivative	% of biliary radioactivity[a]
I	11.0–12.0	17.0–19.0	19.1
IIA	13.5–14.5	18.0–19.5	10.9
III	15.0–16.0	19.5–21.0	13.1
IV	17.5–19.0	23.5–25.0	29.0
V	19.5–20.5	25.5–27.0	17.0
VI	21.0–22.5	28.5–30.0	5.4
total			94.5

[a] Percent of DBCP metabolites excreted within the 6-h collection period. Mean of three rats, unaccounted radioactivity (5.5%) represents unidentified minor metabolites.

were coincubated with rat liver GST (0.2 mg mL^{-1}) and GSH (3 mM) in Tris-HCl buffer (pH 7.7; 100 mM) containing 15 mM sodium citrate and 1 mM EDTA (2 × 5-mL incubations). After incubation at 37 °C for 2 h the enzymatic reaction was terminated by the addition of ice-cold acetone (2 volumes), and the precipitated protein was pelleted by centrifugation (500*g* × 10 min). Acetone was removed from the aqueous phase in vacuo, and the GSH conjugates were converted to their *N*-(benzyloxycarbonyl) derivatives and purified by HPLC as described for the bile samples. The *N*-(benzyloxycarbonyl)-GSH dimethyl ester conjugates were subsequently examined by FAB/MS. In experiments designed to probe hydrolytic mechanisms, sufficient buffer salts were dissolved in $H_2{}^{18}O$ (1 mL) to duplicate the above incubation conditions.

Results

Glutathione conjugates of DBCP either excreted in the bile of rats administered DBCP, or isolated from incubations of rat liver glutathione *S*-transferase with DBCP, were purified by HPLC as their *N*-(benzyloxycarbonyl) dimethyl ester derivatives and analyzed by tandem mass spectrometry. The structures of the GSH conjugates I–IV were elucidated by comparison of their HPLC retention times and CID daughter ion (MS–MS) spectra with those of authentic standards (Table II).

Identification of GSH Conjugates of DBCP Excreted in the Bile of Rats. Following administration of [^{14}C]DBCP (80 mg kg^{-1}) to rats, 19.5 ± 1% of the dose was excreted in the bile within the 6-h collection period. Examination of the biliary GSH conjugates of DBCP by HPLC revealed the presence of five major radioactive metabolites (I–V), which could be separated as their *N*-(benzyloxycarbonyl) derivatives (Figure 1). Upon isolation and methylation, each component displayed a characteristic increase in HPLC retention time (Threadgill et al., 1987; Pearson et al., 1988), associated with an increase in lipophilicity on conversion of the conjugates to their *N*-(benzyloxycarbonyl) dimethyl ester derivatives (Table I). Examination of the purified metabolites by FAB/MS permitted the identification of two classes of GSH conjugates of DBCP. The first class, metabolites I–III, displayed prominent protonated molecular ions (MH^+), but structurally informative fragment ions were absent in the FAB mass spectrum. When examined under CID conditions the MH^+ ions afforded daughter ion spectra, with abundant structurally informative fragment ions that were recognized to be characteristic of simple *S*-alkyl-GSH conjugates of DBCP. The

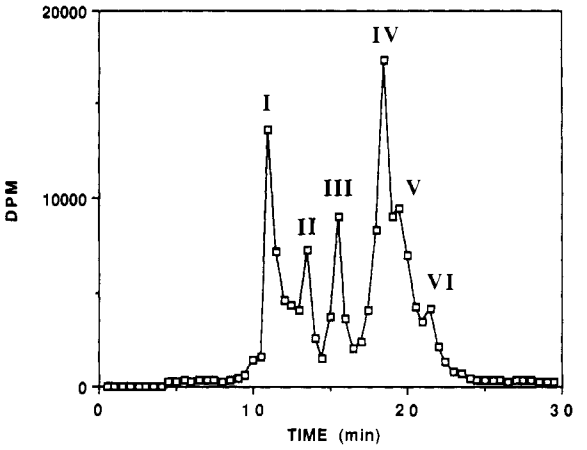
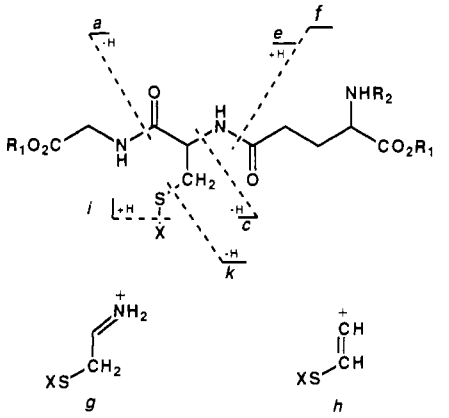


FIGURE 1: HPLC radiochromatogram of biliary glutathione conjugates of DBCP. The metabolites were isolated from bile and separated as their *N*-(benzyloxycarbonyl) derivatives as described under Experimental Procedures.

Chart I: MS–MS Fragmentation of Glutathione Conjugates



second class of GSH conjugates, metabolites IV and V, were structurally more complex than I–III and were substantially more lipophilic; this latter class comprised 46% of the biliary radioactivity and were derived from poly-GSH conjugates of DBCP.

The most polar conjugate, metabolite I, upon FAB/MS analysis displayed an MH^+ ion at *m/z* 544 accompanied by M + Na^+ (*m/z* 566) and M + K^+ (*m/z* 582) adducts (Figure 2A). For each ion, satellite clusters originating from ^{37}Cl and ^{81}Br isotopes were absent, indicating total metabolic loss of DBCP-derived halogen atoms in the genesis of metabolite I. The daughter ion spectrum obtained by low-energy CID (20–40 eV) of the MH^+ ion at *m/z* 544 displayed structurally informative even-electron daughter ions (Figure 2B); the nomenclature used to describe these ions and their proposed origin is depicted in Chart I (Haroldson et al., 1988; Pearson et al., 1988). Three predominant pathways of fragmentation were observed to occur, which in combination account for the majority of the daughter ions observed in the CID spectra of simple *S*-alkyl-GSH conjugates: (i) The first class of fragment ions arose by expulsion of glycine methyl ester (−89 u) to yield ion a at *m/z* 455; the recognition of ion a, combined with elimination of *N*-(benzyloxycarbonyl)-γ-glutamic acid methyl ester to yield structurally informative ion e at *m/z* 267, was consistent with the presence of γ-glutamylcysteinylglycine as a structural element of the MH^+ ion at *m/z* 544. (ii) A second class of daughter ions arose by fragmentation around the cysteinyl thiol; ions a and e underwent further cleavage with loss of 108 u (minus $HSCH_2CHOHCH_2OH$) to yield ions at *m/z* 347 and 159, respectively. The ions at *m/z* 159 and 347

Table II: Daughter Ion Spectra of MH^+ Ions from Glutathione Conjugates of 1,2-Dibromo-3-chloropropane[a]

	substituent X	MH^+	$MH^+ - H_2O$	$MH^+ - CO_2$	$MH^+ - PhCH_2OH$	a	c	e	f	g	h	i	k	other ions					
						Synthetic Standards													
I	$-CH_2CHOHCH_2OH$	544	526 (3)	500 (9)	436[b] (10)	455 (100)	250 (5)	267 (28)	278 (6)	150 (11)		468 (43)	436[b] (10)	411[c] (15)	302[d] (17)	191[e] (5)	181[f] (44)	159[g] (62)	91[h] (8)
IIA	$-CH_2CHOH-CH_3$	528	510 (5)	484 (15)	420 (10)	439 (100)		251 (21)	278 (4)	134 (4)		468 (24)	436 (5)	395[c] (10)	302[d] (16)	191[e] (8)	165[f] (20)	159[g] (47)	91[h] (8)
IIB	$-CH_2CH_2CH_2OH$	528		484 (9)	420 (6)	439 (100)		251 (7)		134 (5)				395[c] (9)	302[d] (8)			159[g] (76)	91[h] (10)
III	$-CH_2CHOHCH_2{}^{35}Cl$	562		518 (11)	454 (7)	473 (100)	268 (8)	285 (31)	278 (5)	168 (18)				429[c] (6)	302[d] (35)		199[f] (15)	159[g] (76)	91[h] (12)
III	$-CH_2CHOHCH_2{}^{37}Cl$	564		520 (8)	456 (6)	475 (100)	270 (5)	287 (21)	278 (6)	170 (10)				431[c] (7)	302[d] (20)		201[f] (5)	159[g] (80)	91[h] (8)
						Biliary Metabolites of DBCP													
I	$-CH_2CHOHCH_2OH$	544	526 (5)	500 (9)	436[b] (10)	455 (100)	250 (5)	267 (28)	278 (6)	150 (11)	133 (5)	468 (43)	436[b] (10)	411[c] (15)	302[d] (17)	191[e] (28)	181[f] (44)	159[g] (96)	91[h] (45)
I	$-CD_2CDOHCD_2OH$	549	531 (5)	505 (10)	441 (6)	460 (100)	255 (5)	272 (24)	278 (15)	155 (15)	138 (4)	468 (31)	436 (5)	416[c] (14)	302[d] (18)	191[e] (25)	186[f] (49)	159[g] (83)	91[h] (40)
II	$-CH_2CHOHCH_3$	528	510 (3)	484 (6)	420 (6)	439 (100)	234 (9)	251 (25)	278 (5)	134 (8)	117 (5)	468 (5)		395[c] (8)	302[d] (24)		165[f] (5)	159[g] (98)	91[h] (9)
II	$-CH_2CHOHCH_3(D_3)$	531		487 (13)	423 (10)	442 (54)	237 (20)	254 (10)		137 (10)	120 (8)			397[c] (11)			168[f] (5)	159[g] (100)	
III	$-CH_2CHOHCH_2{}^{35}Cl$	562	544 (4)	518 (12)	454 (7)	473 (100)		285 (22)	278 (5)	168 (18)		468 (4)		429[c] (5)	302[d] (31)	191[e] (4)	199[f] (4)	159[g] (87)	91[h] (19)
III	$-CD_2CDOHCD_2{}^{35}Cl$	567	549 (4)	523 (8)	459 (7)	478 (100)		290 (21)	278 (6)	173 (10)				434[c] (6)	302[d] (21)		204[f] (4)	159[g] (75)	91[h] (11)
						Glutathione *S*-Transferase Metabolites of DBCP													
I	$-CH_2CHOHCH_2OH$	544	526 (12)	500 (14)	436[b] (20)	455 (100)		267 (12)	278 (8)	150 (5)		468 (28)	436[b] (20)	411[c] (5)	302[d] (37)	191[e] (20)	181[f] (20)	159[g] (56)	
I	$-CD_2CDOHCD_2OH$	549	531 (6)	505 (8)	441 (10)	460 (45)	255 (18)	273 (10)	278 (13)	155 (15)		468 (11)	436 (5)	416[c] (12)	302[d] (10)	191[e] (20)	186[f] (16)	159[g] (100)	91[h] (42)
III	$-CH_2CHOHCH_2{}^{35}Cl$	562		518 (5)	454 (7)	473 (100)	268 (5)	285 (24)	278 (5)	168 (16)				429[c] (6)	302[d] (32)			159[g] (95)	91[h] (9)
III	$-CD_2CDOHCD_2{}^{35}Cl$	567		523 (7)	459 (8)	478 (100)		290 (8)	278 (11)	173 (10)					302[d] (5)			159[g] (100)	91[h] (10)

[a] m/z values and (in parentheses) relative abundances of characteristic daughter ions. Ion abundances are expressed relative to the most intense daughter ion (see Chart I for identifies of ions a–k). [b] Isobaric ions. [c] a – CO_2. [d] $MH^+ - 134 - HSX$. [e] i – X + H. [f] $MH^+ - 363$. [g] e – HSX. [h] $C_7H_7^+$.

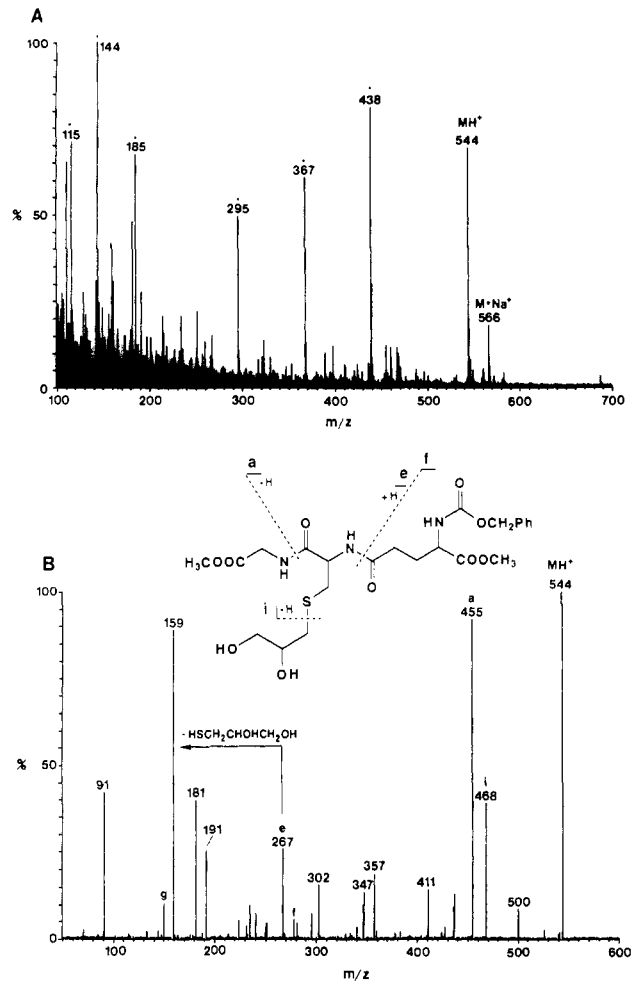


FIGURE 2: (A) Conventional FAB mass spectrum of a derivatized extract of bile collected from a rat administered D_0-DBCP. The MH^+ and $M + Na^+$ ions for *S*-(2,3-dihydroxypropyl)glutathione (I) are evident at *m/z* 544 and 566, respectively. (B) Daughter ion (MS–MS) spectrum obtained by collision activation of the MH^+ ion (at *m/z* 544) of derivatized *S*-(2,3-dihydroxypropyl)glutathione (I) isolated from the bile of a rat administered D_0-DBCP. The structure of the metabolite and the proposed origins of the daughter ions are as indicated. Collision-induced dissociation was performed in the first (rf only) quadrupole (*q*) of a VG 70-SEQ hybrid tandem mass spectrometer (EBqQ geometry) with argon as a collision gas (2×10^{-6} Torr) and collision energies of 40 eV.

are independent of the substituents on the *S*-alkyl component of the GSH conjugate and thus provide details of the nature of the conjugated xenobiotic moiety. Additional fragmentation at the thiol with charge retention on the GSH moiety and proton transfer to the xenobiotic moiety gave rise to ion i at *m/z* 468 with expulsion of the elements of propanediol from the MH^+ ion. A similar cleavage was also observed from ion e to yield a prominent daughter ion at *m/z* 181, an observation, which in common with ion i, is consistent with the attachment of a dihydroxypropyl moiety to the cysteinyl thiol. (iii) The third class of CID-induced fragmentation was characterized by the expulsion of small neutral fragments, which were ascribed to reactions involving the methyl ester (minus CO_2) or aryloxycarbonyl functionalities (minus $PhCH_2OH$). Therefore, the daughter ion spectra of simple *S*-alkyl-GSH conjugates provide an intimate knowledge of the nature of the drug residue, the GSH moiety, and the functionalized amino and carboxyl groups of the conjugate. A comparison of the CID daughter ion spectrum of DBCP metabolite I with a CID daughter ion spectrum of authentic *N*-(benzyloxycarbonyl)-*S*-(2,3-dihydroxypropyl)glutathione dimethyl ester (Table II) confirmed the identity of metabolite I. Moreover, examination of *S*-(2,3-dihydroxypropyl)glutathione by HPLC as either an *N*-(benzyloxycarbonyl) derivative or an *N*-(benzyloxycarbonyl) dimethyl ester derivative established retention times for derivatives of the standard identical with those observed for the corresponding derivative of biliary metabolite I.

The recognition of characteristic daughter ions arising by expulsion of the derivatized glycine (−89 u) and glutamate residues (−277 u) and subsequent loss of the xenobiotic moiety accompanied by SH to afford common ions at *m/z* 159 and 347 facilitate the identification of simple *S*-alkyl-GSH conjugates of DBCP. Metabolite II exhibited HPLC properties characteristic of GSH conjugates (Table I) and upon FAB/MS analysis displayed prominent ions at *m/z* 528 (MH^+), 550 ($M + Na^+$), and 566 ($M + K^+$; data not shown). A daughter ion spectrum of the MH^+ ion at *m/z* 528 recorded under CID conditions (Table II) displayed ions a (−89 u) and e (−277 u), with the subsequent loss of 92 u (minus $HSCH_2CHOHCH_3$) from ions a and e, fragments consistent with a metabolite whose structure may be either *S*-(2-hydroxypropyl)glutathione (IIA) or *S*-(3-hydroxypropyl)glutathione (IIB). Comparison of the daughter ion spectrum and HPLC mobility of *N*-(benzyloxycarbonyl)-*S*-(2-hydroxypropyl)glutathione dimethyl ester confirmed IIA to be metabolite II.

In a manner similar to that of metabolites I and II, metabolite III displayed an increase in HPLC retention time upon methylation, and when examined by FAB/MS, prominent ions at *m/z* 562 (MH^+), 584 ($M + Na^+$), and 600 ($M + K^+$) were observed (see supplementary material, see paragraph at end of paper regarding supplementary material). The striking feature of the MH^+ ion at *m/z* 562 was the presence of a ^{37}Cl satellite at *m/z* 564 in an approximate ratio of 3:1 (562:564), when corrected for matrix background and the ^{34}S contribution to *m/z* 564. The ^{37}Cl satellite was also present for the $M + Na^+$ and $M + K^+$ pseudo molecular ions. Examination of the daughter ion spectrum obtained upon CID of the MH^+ ion at *m/z* 562 (^{35}Cl satellite) revealed a series of structurally informative even-electron daughter ions similar to those previously described for *S*-(2,3-dihydroxypropyl)glutathione, with an appropriate shift in *m/z* value for ions retaining the xenobiotic residue (e.g., ions a, e, g, and h). A CID spectra of the ^{35}Cl satellite (*m/z* 562) of authentic *N*-(benzyloxycarbonyl)-*S*-(3-chloro-2-hydroxypropyl)glutathione dimethyl ester displayed a daughter ion spectrum identical with that of metabolite III (Table II). Comparison of HPLC retention times of the two derivatized forms of metabolite III served to confirm the structure of metabolite III as *S*-(3-chloro-2-hydroxypropyl)glutathione.

Metabolites IV and V differed significantly from I–III, in both HPLC retention times and mass spectral properties. The principle biliary metabolite of DBCP, metabolite IV, displayed an increase in retention time upon treatment with methanolic HCl and was substantially more lipophilic than the previous simple *S*-alkylglutathione conjugates. In contrast to the spectra of metabolites I–III, examination of the FAB mass spectrum of IV revealed a series of ions between *m/z* 600 and *m/z* 1000 (Figure 3A). The spectrum, while complex, provided a significant amount of structural information and indicated the presence of two components: derivatized GSSG and a diglutathionyl conjugate of DBCP which were sufficiently similar to comigrate under the HPLC conditions employed. The recognition of structurally informative fragment ions in the FAB/MS spectrum of metabolite IV is aided by

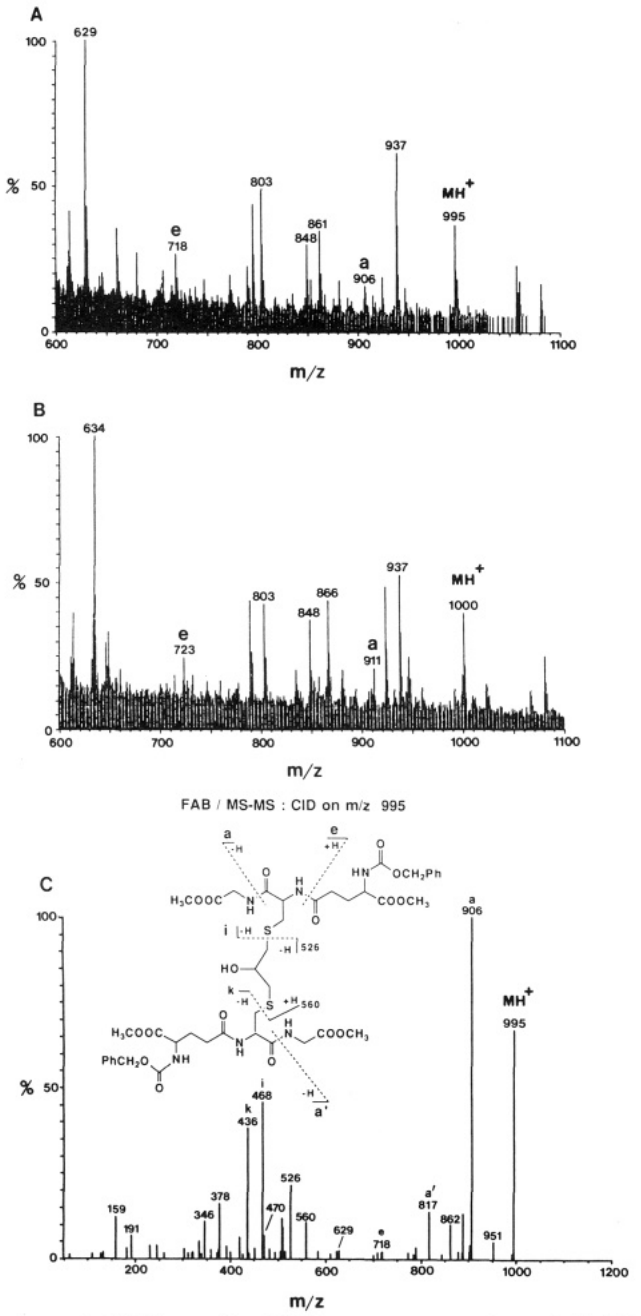


FIGURE 3: (A) Conventional FAB mass spectrum of metabolite IV isolated from the bile of a rat administered D_0-DBCP. The MH^+ ion of IV (at m/z 995) and structurally informative fragment ions arising from loss of glutamate (ion e at m/z 718) or glycine (ion a at m/z 906) moieties are as indicated. (B) Conventional FAB mass spectrum of metabolite IV isolated from the bile of a rat administered D_5-DBCP. The MH^+ ion of IV (at m/z 1000) and structurally informative fragment ions arising from loss of glutamate (ion e at m/z 723) or glycine (ion a at m/z 911) moieties are as indicated. (C) Daughter ion (MS–MS) spectrum obtained by collision activation of the MH^+ ion (at m/z 995) of derivatized IV isolated from rat bile. The structure of the metabolite and the proposed origins of the major daughter ions are as indicated. Collision-induced dissociation (CID) was performed in the first (rf only) quadrupole (q) of a VG 70-SEQ hybrid tandem mass spectrometer (EBqQ geometry), with argon as a collision gas (7×10^{-6} Torr) and collision energies of 35 eV.

comparison with the spectrum obtained from metabolite IV derived from D_5-DBCP, in which DBCP-related fragment ions were shifted by a positive mass increment of 5 u, which provided information on deuterium content in addition to the identity of the metabolite (Figure 3B). The MH^+ ion of metabolite IV (m/z 995) is associated with ions arising from losses of glycine methyl ester (m/z 906; ion a), of the benzyloxycarbonyl functionality (−134 u), and of derivatized γ-glutamate (m/z 718; ion e). The e ion also underwent a loss of 89 u to yield the ion at m/z 629. The ion at m/z 937 is unrelated to DBCP as, in the mass spectrum of D_5-DBCP-derived metabolite IV, it remained unchanged in mass and may be assigned as the MH^+ of derivatized oxidized glutathione (GSSG). This inference is supported by the appearance of ions at m/z 848 (GSSG − glycine methyl ester; ion a) and m/z 803 (GSSG − benzyloxycarbonyl functionality).

The MS–MS daughter ion spectra obtained by CID of either m/z 937 or m/z 995 displayed a series of structurally informative daughter ions. The three basic modes of fragmentation observed for *S*-(2,3-dihydroxypropyl)glutathione were also operative for the higher mass di-GSH conjugate IV. The CID spectrum was characterized by incremental losses of 89 u, corresponding to the loss of two glycine methyl ester functionalities to yield ions a and a′, an observation consistent with the presence of two glycine components. Additional cleavages from the loss of the derivatized glutamate moiety affords ion e at m/z 718, and further cleavages adjacent to the two sulfur atoms yielded ions at m/z 436, 468, 526, and 560 (Figure 3C). The ions observed below 400 mass units arise via fragmentation of either of the GSH components of IV as evidenced by a comparison with the CID spectrum of *S*-(2,3-dihydroxypropyl)glutathione (Figure 2B) in which abundant ions of the same mass were observed. The origin of these low mass ions is described in detail in Table II. The structural information thus derived from FAB/MS and FAB/MS–MS is consistent with the formation of a diglutathionyl conjugate, 1,3-di(*S*-glutathionyl)propan-2-ol (IV), or a regioisomeric metabolite, 1,2-di(*S*-glutathionyl)propan-3-ol. Comparison of the HPLC retention times and the CID daughter ion spectra of an authentic sample of 1,3-di(*S*-glutathionyl)propan-2-ol supports either of these proposals.

Mass spectral analysis of metabolite V revealed prominent ions at m/z 852 (MH^+) and m/z 874 ($M + Na^+$) which may arise from 1-(glycyl-*S*-cysteinyl)-3-(*S*-glutathionyl)propan-2-ol as its dibenzyloxycarbonyl trimethyl ester derivative. We propose that this metabolite arises by partial enzymatic degradation of 1,3-di(*S*-glutathionyl)propan-2-ol by the γ-glutamyl transpeptidase of biliary ductular epithelium (Abbott & Meister, 1986). The FAB/MS spectrum of V displayed two other intense ions at m/z 794 (MH^+) and m/z 816 ($M + Na^+$), which arise from the dibenzyloxycarbonyl trimethyl ester derivative of the mixed disulfide of glutathione and cysteinylglycine; in a manner similar to that of V, this also arises by partial γ-glutamyl transpeptidase cleavage of GSSG and displays HPLC properties indistinguishable from those of metabolite V.

Identification of GSH Conjugates of D_5-DBCP Excreted in the Bile of Rats. The mechanistic details derived from deuterium retention in the biliary GSH conjugates excreted in the bile of rats administered D_5-DBCP, in addition to aiding in the identification of metabolites of DBCP as exemplified for 1,3-di(*S*-glutathionyl)propan-2-ol (IV, Figure 3A,B), also provided an insight into the nature of the transient species which may serve as an intermediate in the biogenesis of the GSH conjugates of DBCP.

Isolation of *S*-(2,3-dihydroxypropyl)glutathione and mass spectral analysis following administration of D_5-DBCP revealed ions at m/z 549 (MH^+), 571 ($M + Na^+$), and 587 ($M + K^+$; Figure 4B). The daughter ion spectrum obtained by CID upon m/z 549 was similar to that obtained for m/z 544, with a shift in m/z by a positive mass increment of 5 u for

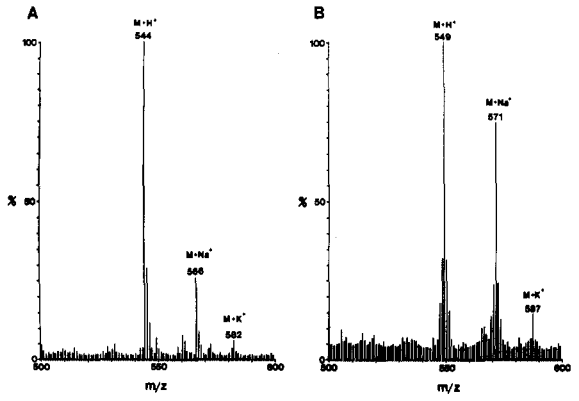


FIGURE 4: (A) Partial FAB mass spectrum of derivatized metabolite I isolated from rat bile. The MH^+ ion (at m/z 544) of the D_0-DBCP-derived metabolite and its satellite cluster arising from natural abundance isotopes are evident. (B) Molecular ion region of derivatized metabolite I isolated from rat bile. The MH^+ ion (at m/z 549) of the D_5-DBCP-derived metabolite and its isotopic enrichment with deuterium are evident.

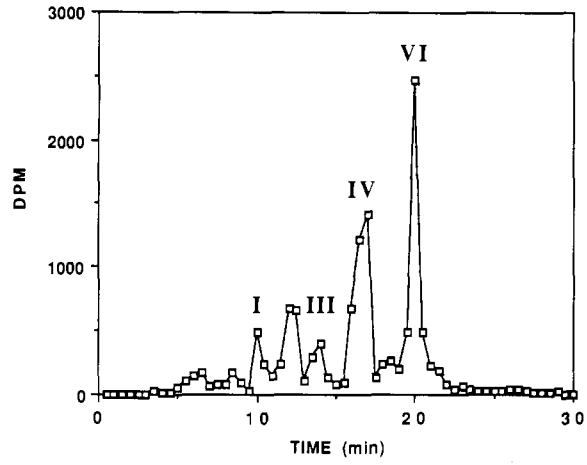


FIGURE 5: HPLC radiochromatogram of glutathione conjugates of DBCP formed in incubations of rat liver glutathione transferase fortified with GSH (3 mM) and DBCP (1 mM). The metabolites were isolated and separated as their *N*-(benzyloxycarbonyl) derivatives as described under Experimental Procedures.

ions derived by fragmentation pathways not involving loss of the xenobiotic moiety (Table II). Additional ions common to the spectrum of the metabolite derived from D_0-DBCP and D_5-DBCP were m/z 347 (ion a – $HSCH_2CHOHCH_2OH$ or $HSC^2H_2C^2HOHC^2H_2OH$) and m/z 159 (ion e – $HSCH_2CHOHCH_2OH$ or $HSC^2H_2C^2HOHC^2H_2OH$). The isotope cluster in the FAB mass spectrum indicates complete retention of all five deuteriums (Figure 4B; based on 85 atom % excess D_5-DBCP as substrate), and the similarities between the CID spectra for the D_0 and D_5 metabolites (Table II) indicate a common structure. In a similar fashion examination of *S*-(3-chloro-2-hydroxypropyl)glutathione (III) by FAB/MS following administration of D_5-DBCP revealed the presence of a molecular ion at m/z 567 and a ^{37}Cl satellite at m/z 569 (see supplementary material). Metabolites IV (Figure 3A,B) and V (data not shown) both display quantitative retention of deuterium, an observation that is consistent with the previous contention that metabolite V is derived by γ-glutamyl transpeptidase hydrolysis of metabolite IV.

The deuterium retention in metabolites I, III, IV, and V suggests a common mechanism of formation, which is independent of C–H/C–D bond cleavage and as a consequence does not involve a cytochrome P-450 generated intermediate. In contrast, FAB mass spectral analysis of *S*-(2-hydroxypropyl)glutathione (IIA) following administration of D_5-DBCP revealed retention of only three deuterium atoms (MH^+, m/z 531). In this instance comparison of the CID daughter ion spectra of *S*-(2-hydroxypropyl)glutathione (IIA) derived from either D_5-DBCP (MH^+, m/z 531) or D_0-DBCP (MH^+, m/z 528) displayed the characteristic ions arising by loss of glycine methyl ester (–89 u; ion a) and derivatized γ-glutamic acid (–277 u; ion e) which were shifted by an increment of three mass units (Table II).

Glutathione S-Transferase Mediated Metabolism of DBCP. The in vivo findings indicate that the major route of metabolism of DBCP to hydroxylated alkyl-GSH conjugates occurred via pathways independent of cytochrome P-450, as evidenced by quantitative deuterium retention in metabolites I, III, IV, and V. In order to evaluate the catalytic potential for glutathione *S*-transferase to generate GSH conjugates of DBCP with quantitative retention of deuterium, an approximately equimolar mixture of D_0-DBCP and D_5-DBCP was incubated with rat liver glutathione *S*-transferase, and the resultant GSH conjugates were isolated as described for the in vivo experiments. Examination of the *N*-(benzyloxycarbonyl)-GSH conjugates by HPLC revealed three major metabolites whose HPLC mobility (Figure 5) corresponded to metabolites I, III, and IV observed in vivo. Subsequent examination of metabolite I by FAB/MS revealed the presence of a twin ion isotope cluster at m/z 544 and 549 corresponding to the MH^+ ions of unlabeled and $^2H_5$-labeled *N*-(benzyloxycarbonyl)-*S*-(2,3-dihydroxypropyl)glutathione dimethyl ester, respectively (Figure 6A). The reduced intensity of m/z 549 compared with m/z 544 is due to the presence of a significant (15 atom % excess) population of [$^2H_4$]GSH conjugate derived from the partially enriched D_5-DBCP substrate in the mixture. Examination of *S*-(3-chloro-2-hydroxypropyl)glu-

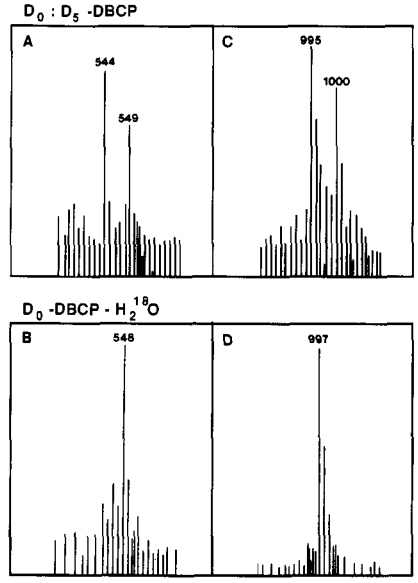


FIGURE 6: (A) Partial FAB mass spectrum illustrating the molecular ion region of metabolite I isolated from a GST-catalyzed incubation of D_0-DBCP and D_5-DBCP with GSH. The MH^+ ions of the D_0- and D_5-DBCP-derived metabolites are evident at m/z 544 and 549, respectively. (B) Molecular ion region of metabolite I isolated from a GST-catalyzed incubation of D_0-DBCP with GSH in $H_2{}^{18}O$. The MH^+ ion of the D_0 metabolite enriched with two atoms of ^{18}O is evident at m/z 548. (C) Molecular ion region of metabolite IV isolated from a GST-catalyzed incubation of D_0-DBCP and D_5-DBCP with GSH. The MH^+ ions of the D_0- and D_5-DBCP-derived metabolites are evident at m/z 995 and 1000, respectively. (D) Molecular ion region of metabolite IV isolated from a GST-catalyzed incubation of D_0-DBCP with GSH in $H_2{}^{18}O$. The MH^+ ion of the D_0-DBCP metabolite enriched with one atom of ^{18}O is evident at m/z 997.

tathione (III) by FAB/MS revealed isotope clusters at m/z 562 and 567, in a ratio similar to that observed for metabolite I but also accompanied by ^{37}Cl satellites (not shown) indicative of formation *S*-(3-chloro-2-hydroxypropyl)glutathione (III) with full retention of deuterium. Metabolite IV displayed a cluster of ions at m/z 995 and 1000 in a ratio similar to that observed for metabolites I and III (Figure 6C). Therefore, the generation of metabolites I, III, and IV by glutathione transferase, with quantitative retention of deuterium, suggests a common mechanism of formation of these metabolites both in vivo and in vitro which does not require C–H/C–D bond cleavage and as a consequence is independent of cytochrome P-450. A plausible explanation for the observed pattern of metabolites and their genesis with full retention of deuterium centers upon GST-catalyzed conjugation of DBCP with GSH. The subsequent spontaneous episulfonium ion formation with intramolecular displacement of chlorine or bromine results in the formation of a transient reactive species. The ultimate fate of this species, i.e., hydrolysis by water or alkylation of GSH, dictates the pattern of metabolites observed. To explore the involvement of hydrolytic mechanisms in the formation of the GSH conjugate of DBCP in vitro, the GST incubations were repeated with D_0-DBCP as a substrate, in an environment enriched in $H_2{}^{18}O$ water and the ^{18}O incorporation into *S*-(2,3-dihydroxypropyl)glutathione (I) and 1,3-di(*S*-glutathionyl)propan-2-ol (IV) was determined by FAB/MS. Examination of metabolite I formed under these conditions (Figure 6B) demonstrated a shift in mass of 4 u for the MH^+ ion at m/z 544 to m/z 548, indicative of incorporation of two atoms of ^{18}O; conversely, metabolite IV incorporated one ^{18}O atom (Figure 6D). These observations are consistent with the genesis of metabolite I via the intermediacy and subsequent hydrolysis of two episulfonium ion intermediates. In an analogous manner metabolite IV is formed from two episulfonium ion intermediates with one hydrolytic step and alkylation of GSH by a second episulfonium ion. The major metabolite formed in the incubations of DBCP with GST was examined by FAB/MS and displayed an MH^+ ion at m/z 1447, an observation consistent with the successive alkylation of GSH by two episulfonium ion intermediates to afford a trisubstituted GSH conjugate metabolite of DBCP. Therefore, the pattern of metabolites and their status of isotopic enrichment observed in a variety of incubations of DBCP with GST are consistent with the intermediacy of two transient reactive episulfonium ions. It is interesting to note that the late-eluting triglutathionyl conjugate metabolite of DBCP was also detected by HPLC in rat bile (Figure 1, t_r = 21–22.5 min, VI) but at levels insufficient for satisfactory mass spectrometric characterization.

The absence of 1-(glycyl-*S*-cysteinyl)-3-(*S*-glutathionyl)-propan-2-ol in GST incubations is in accord with the proposed mechanism of its genesis in vivo, in which 1,3-di(*S*-glutathionyl)propan-2-ol is degraded enzymatically by γ-glutamyl transpeptidase. The genesis of *S*-(2-hydroxypropyl)glutathione in vivo involves an oxidative step with C–H/C–D bond cleavage, a process not expected to occur in incubations of glutathione transferase with DBCP, and hence, *S*-(2-hydroxypropyl)glutathione is not observed in vitro, in incubations that lack cytochrome P-450 activity.

Discussion

The biochemical events in the formation of GSH conjugate metabolites of vicinally dihalogenated alkanes may represent either a bioactivation mechanism or alternatively may serve in the detoxification of cytochrome P-450 generated reactive intermediates. Reactive intermediates generated by either of

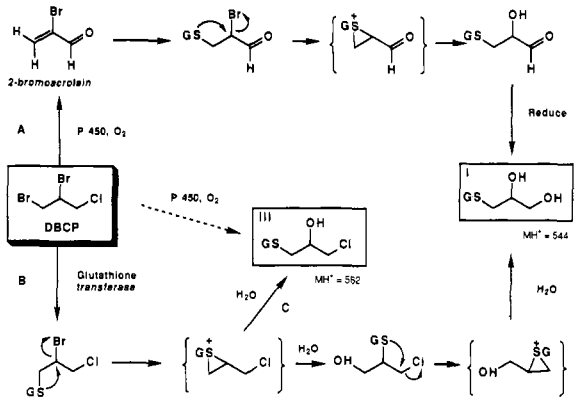


FIGURE 7: Metabolic scheme for the biotransformation of DBCP to GSH conjugates in vivo. The scheme illustrates potential pathways of formation of I via the intermediacy of either 2-bromoacrolein (pathway A) or an episulfonium ion (pathway B) intermediate(s). In accord with the experimental findings, metabolite III may also be formed by branching (pathway C) of the episulfonium ion pathway implicated in the genesis of metabolite I.

these pathways may be responsible for DNA damage and/or cytotoxicity; therefore, an understanding of the relative importance of each pathway is germane to elucidating the biochemical events in the activation of haloalkanes. The bioactivation pathway is exemplified by GST-catalyzed conjugation of 1,2-dibromoethane, which is a prerequisite for 1,2-dibromoethane binding to the N-7 position of guanine bases in DNA (Koga et al., 1986; Ozawa & Guengerich, 1983) and proceeds via the intermediacy of an electrophilic episulfonium ion (Peterson et al., 1988). Metabolic studies conducted in vivo have demonstrated that the ultimate urinary metabolite *N*-acetyl-*S*-(2-hydroxyethyl)cysteine was observed to originate either from 2-bromoacetaldehyde or via GSH conjugation pathways in a ratio of 4:1, respectively (Van Bladeren et al., 1981). Moreover, it has been proposed that the genotoxicity associated with 1,2-dibromoethane is mediated primarily via conjugation with GSH (White et al., 1983). Although episulfonium ions may be formed chemically (Dohn & Casida, 1987; Henkel & Amato, 1988), their propensity to hydrolyze under physiological conditions only permits indirect visualization of these transient species by observation of stereochemical events in their biogenesis (Peterson et al., 1988) or by use of stable isotopes as mechanistic probes. In the present investigation we have identified six glutathione conjugate metabolites of DBCP by MS–MS techniques and addressed their mechanisms of formation both in vivo and in vitro, by use of a perdeuterio analogue of DBCP.

The major *S*-alkyl-GSH conjugate metabolite of DBCP, viz., *S*-(2,3-dihydroxypropyl)glutathione, may arise, at least in principle, by two distinct but convergent metabolic pathways. The major mutagenic metabolite of DBCP has been identified as 2-bromoacrolein (Omichinski et al., 1988a), which originates by cytochrome P-450 oxidation at either C_1 or C_3 of DBCP, followed by two spontaneous dehydrohalogenations. In principle, conjugate addition of GSH to 2-bromoacrolein with hydrolysis of the intermediate episulfonium ion derived from *S*-(2-bromo-3-oxopropyl)glutathione and subsequent reduction of the aldehyde functionality may yield *S*-(2,3-dihydroxypropyl)glutathione (Figure 7, pathway A) in a mechanism involving loss of two hydrogen (or deuterium) atoms from DBCP (or D_5-DBCP). Alternatively, formation of *S*-(2,3-dihydroxypropyl)glutathione by a glutathione *S*-transferase reaction with subsequent hydrolysis of two successive episulfonium ion intermediates would afford *S*-(2,3-dihydroxypropyl)glutathione without C–H/C–D bond cleavage

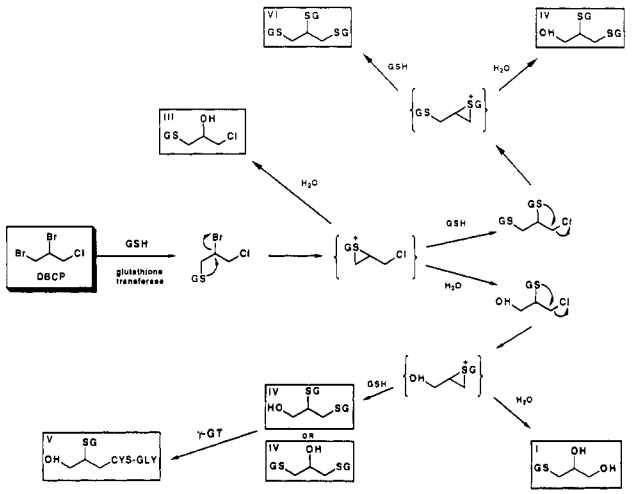


FIGURE 8: Overall metabolic activation scheme for the biotransformation of DBCP to GSH conjugates. This unified pathway involving three different episulfonium ion intermediates accounts for 88.4% of the biliary metabolites and is entirely consistent with the isotopic enrichment of the metabolites derived from D_5-DBCP or formed metabolically in incubations enriched in $H_2{}^{18}O$.

(Figure 7, pathway B). Mass spectral analysis of *S*-(2,3-dihydroxypropyl)glutathione derived from D_5-DBCP in vivo displayed net deuterium retention, an observation consistent with the formation of *S*-(2,3-dihydroxypropyl)glutathione exclusively by a glutathione *S*-transferase catalyzed pathway. In a manner similar to *S*-(2,3-dihydroxypropyl)glutathione formation, *S*-(3-chloro-2-hydroxypropyl)-*N*-acetylcysteine, the presumed ultimate urinary metabolite of *S*-(3-chloro-2-hydroxypropyl)glutathione, has been proposed to originate either from 1-bromo-3-chloroacetone (Dohn et al., 1988) or via the intermediacy of an episulfonium ion (Dohn et al., 1988; Gingell et al., 1987). The former pathway involves oxidation at C_2 and involves metabolic elimination of a hydrogen (or deuterium) atom at the C_2 positions of DBCP. We have found that, upon administration of D_5-DBCP to rats, *S*-(3-chloro-2-hydroxypropyl)glutathione displayed quantitative retention of deuterium, strongly suggesting that *S*-(3-chloro-2-hydroxypropyl)glutathione is formed metabolically by a pathway independent of cytochrome P-450.

In previous investigations (Dohn et al., 1988) employing heteronuclear NMR spectroscopy it was proposed, erroneously, that *S*-(2,3-dihydroxypropyl)glutathione and *S*-(3-chloro-2-hydroxypropyl)glutathione may arise by detoxication of P-450-generated reactive intermediates. Indeed, the approach of using a ^{13}C-labeled analogue of DBCP failed to identify 1,3-di(*S*-glutathionyl)propan-2-ol and was incapable of visualizing the metabolic precursors to *S*-(2,3-dihydroxypropyl)-glutathione or *S*-(3-chloro-2-hydroxypropyl)glutathione. In the present investigation we propose that both *S*-(2,3-dihydroxypropyl)glutathione and *S*-(3-chloro-2-hydroxypropyl)glutathione originate via glutathione transferase mediated conjugation of glutathione with DBCP at C_1 to yield *S*-(3-chloro-2-bromopropyl)glutathione (Figure 8). The availability of a leaving group in the β-position promotes episulfonium ion formation (Schasteen & Reed, 1983; Foureman & Reed, 1987), which upon hydrolysis at C_2 yields *S*-(3-chloro-2-hydroxypropyl)glutathione which is presumed to be a final stable metabolite. An alternative fate for the episulfonium ion formed from *S*-(3-chloro-2-bromopropyl)glutathione is hydrolysis at C_1 to yield the (β-haloalkyl)glutathione conjugate *S*-(1-hydroxy-3-chloropropyl)glutathion-2-yl, which may spontaneously form a second episulfonium ion; subsequent hydrolysis or alkylation of GSH by this intermediate would afford *S*-(2,3-dihydroxypropyl)glutathione or 1,3-di(*S*-glutathionyl)propan-2-ol, respectively, with full deuterium retention. This proposed mechanism may of course yield regioisomeric metabolites, which at present cannot be distinguished by MS–MS techniques.

The role of glutathione transferase in the formation of *S*-(2,3-dihydroxypropyl)glutathione, *S*-(3-chloro-2-hydroxypropyl)glutathione, and 1,3-di-(*S*-glutathionyl)propan-2-ol is corroborated by the identification of these metabolites generated in incubations of rat liver glutathione transferase with DBCP and GSH. Quantitative deuterium retention in metabolites derived from D_5-DBCP and incorporation of ^{18}O from $H_2{}^{18}O$ into each of these metabolites are consistent with hydrolysis of an episulfonium ion derived from the putative *S*-(3-chloro-2-bromopropyl)glutathione. We consider that *S*-(2,3-dihydroxypropyl)glutathione, *S*-(3-chloro-2-hydroxypropyl)glutathione, and 1,3-di(*S*-glutathionyl)propan-2-ol represent the final stable metabolites from the bioactivation of DBCP and are unlikely to be cytotoxic or mutagenic. By way of analogy it has been demonstrated that *S*-(2-hydroxyethyl)cysteine and *S*-(3-chloropropyl)cysteine do not appear to form episulfonium ion intermediates and are not nephrotoxic either in vivo (Elfarra et al., 1985) or in vivo (Webb et al., 1987). Moreover, a model β-bromopropyl-substituted mercapturic acid, *N*-acetyl-*S*-(2-bromopropyl)cysteine methyl ester, was found to be mutagenic to *Salmonella typhimurium* TA 100 (Zoetemelk et al., 1986) and induced a number of revertants similar to that observed with the corresponding mercapturate of 1,2-dibromoethane (van Bladeren et al., 1980).

The bioactivation of 1,2-dibromoethane to an episulfonium ion intermediate, in a GSH-dependent process, that binds to DNA requires the minimal structural features of a vicinal dibromopropyl functionality. The interaction of DBCP metabolites with DNA when activated by liver fractions, however, appears to be more complex as significant microsomally mediated binding of DBCP to DNA has been observed (Inskeep & Guengerich, 1984). Furthermore, DNA damage in isolated hepatocytes exposed to DBCP or D_5-DBCP was subject to a kinetic isotope effect, indicating the presence of a significant P-450-dependent component in addition to a glutathione-mediated activation pathway (Holme et al., 1989). The role of 2-bromoacrolein in the mutagenesis of DBCP has been established (Omichinski et al., 1988a), and its potential to form cyclic adducts with deoxyguanosine (Meerman et al., 1987) may account for the deuterium-sensitive component of the DNA damage in hepatocytes.

DNA strand breaks are induced by DBCP in spermatogenic cells; these are insensitive to deuterium substitution, display a requirement for glutathione, and are independent of P-450. In a similar fashion renal necrosis and DNA damage induced by DBCP in vivo were not shown to be altered when selectively deuterated analogues of DBCP were administered (Omichinski et al., 1988b). Both the kidneys and testes display high glutathione transferase activity toward DBCP (Miller et al., 1986), which supports the hypothesis that DBCP induces DNA damage in extrahepatic target organs by a glutathione-dependent activation pathway. The identification of GSH conjugates of DBCP in vivo and in vitro that are formed with full detuerium retention from D_5-DBCP, and which are formed by $H_2{}^{18}O$ hydrolysis in vitro, is consistent with the formation of an episulfonium ion intermediate.

In conclusion, we have presented compelling chemical evidence for glutathione-dependent bioactivation of DBCP to reactive episulfonium ion intermediates which may be implicated in the expression of DBCP-induced extrahepatic

target-organ toxicity and DNA damage. Identification of GSH conjugate metabolites of DBCP and elucidation of their mechanism of formation facilitate the recognition of transient intermediates and prediction of their likely interaction with constituents of nucleic acids. Investigations are currently in progress to identify covalent adducts to nucleic acids arising from episulfonium intermediates and to establish the importance of glutathione-dependent activation of DBCP in preparations of isolated kidney and testicular cells.

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Supplementary Material Available

Three figures depicting FAB/MS–MS (CID) daughter ion spectra of authentic *S*-(2,3-dihydroxypropyl)glutathione in support of the identification of metabolite I and mass spectral data for the identification of *S*-(3-chlorohydroxypropyl)glutathione (III) derived metabolically from DBCP or D_5-DBCP, illustrating deuterium isotope incorporation and structural characterization from the FAB/MS–MS (CID) daughter ion spectra (3 pages). Ordering information is given on any current masthead page.

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