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METABOLITES OF [3-^{13}C]1,2-DIBROMO-3-CHLOROPROPANE IN MALE RATS STUDIED BY ^{13}C AND ^{1}H-^{13}C CORRELATED TWO-DIMENSIONAL NMR SPECTROSCOPY

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Abstract—Metabolism of 1,2-dibromo-3-chloropropane (DBCP) was examined by direct ^{13}C and ^{1}H-^{13}C correlated two-dimensional NMR spectroscopy of bile and urine of male albino rats treated intraperitoneally with [3-^{13}C]DBCP at 81 mg/kg. The 3-^{13}C label was introduced at 99% enrichment by coupling [^{13}C]paraformaldehyde with vinyllithium to give [1-^{13}C]allyl alcohol which was converted to allyl chloride with carbon tetrachloride/triphenylphosphine and then brominated. Fifteen ^{13}C NMR signals were observed for biliary metabolites and twelve for urinary metabolites. Nine of the biliary metabolite ^{13}C NMR signals were very similar or identical to those for nine urinary metabolites. The DBCP-derived moieties of five metabolites were identified by comparison of their ^{13}C NMR chemical shifts, ^{13}C multiplicities [obtained via the distortionless enhancement by polarization transfer (DEPT) pulse sequence], and chemical shifts of the directly-attached protons (obtained via two-dimensional NMR) with those of authentic standards. They were *E*- and *Z*-$RSCH_2CH{=}{}^{13}CHCl$, $RSCH_2CHOH{}^{13}CH_2Cl$, $RSCH_2CHOH{}^{13}CH_2OH$ and $RS{}^{13}CH_2CHOHCH_2OH$, where R is probably glutathionyl in bile and *N*-acetylcysteinyl in urine. The mechanism proposed for formation of both the *E*- and *Z*-isomers of $RSCH_2CH{=}{}^{13}CHCl$ involves radical-initiated dehydrobromination followed by reaction of the intermediate allylic bromides with glutathione (GSH). The $RSCH_2CHOHCH_2Cl$ conjugate may arise from direct GSH conjugation and hydrolysis of the secondary bromine via a thiiranium ion intermediate. The proposed origin of the $RSCH_2CHOHCH_2OH$ conjugate labeled at either carbon-1 or carbon-3 is oxidation of DBCP at the bromomethyl or chloromethyl substituent, respectively, followed by two spontaneous dehydrohalogenations to give the highly reactive 2-bromopropenal, and addition of GSH followed by reduction of the aldehyde functionality. An alternative mechanism for the formation of the $RSCH_2CHOHCH_2Cl$ and $RSCH_2CHOHCH_2OH$ derivatives involves carbon-2 oxidation to give 1-bromo-3-chloroacetone followed by reaction with GSH and reduction of the ketone functionality with or without hydrolysis of the chloro substituent. 2-Bromopropenal, 1-bromo-3-chloroacetone, or GSH conjugates derived from these intermediates may be involved in the male reproductive toxicity, nephrotoxicity and genotoxicity of DBCP.

DBCP† was one of the major soil fumigants and nematocides but its use is no longer permitted in the United States because of adverse toxicological effects including disruption of the male reproductive system, liver and kidney damage, and carcinogenesis [1]. Workers involved in DBCP formulation suffered from a high incidence of azospermia (complete absence of viable sperm) and oligospermia (greatly reduced sperm counts) [1, 2]. The unique effect on sperm production was reproduced in rats and rabbits, confirming that DBCP was the causative agent and suggesting the existence of a critical target in the male reproductive system [3–7]. The kidney in rats is more sensitive than the liver to damage by acute doses of DBCP [4]. Technical grade DBCP is a potent carcinogen on inhalation by rats and mice, although the contribution of an epichlorohydrin additive is unknown [8]. However, purified DBCP is genotoxic in a number of short-term mutagenesis assays [1].

The diverse toxic effects of DBCP probably result from one or more bioactivation products since, in general, halogenated alkanes exert their toxicity via metabolites formed oxidatively (through cytochrome P-450-mediated reactions) or by conjugation with GSH (e.g. via sulfur mustards). DBCP requires metabolic activation to show mutagenic activity in the Ames' *Salmonella* assay [1, 9, 10]. It undergoes covalent binding *in vitro* and *in vivo* [11, 12] and is metabolized to CO_2 and many polar metabolites that are excreted in urine and feces [11, 13]. Some of the metabolites may be GSH conjugates because DBCP is a potent depleter of liver GSH, causing the loss within 2–4 hr of ~0.5 mol of GSH for every mol of administered DBCP [14, 15].

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† Abbreviations: ^{13}Cs, ^{13}carbons; DBCP, 1,2-dibromo-3-chloropropane; 2-D NMR, ^{1}H-^{13}C correlated two-dimensional NMR spectroscopy; DMSO, dimethyl sulfoxide; DMSO-d_6, perdeuterated dimethyl sulfoxide; GSH, glutathione; ^{1}Hs, protons; ^{2}Hs, deuteriums; i.p., intraperitoneal; s.c., subcutaneous; TTP, 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropanoic acid; and DEPT, distortionless enhancement by polarization transfer.

This report identifies several metabolites of [3-^{13}C]DBCP in the bile and urine of male rats by direct ^{13}C and 2-D NMR spectroscopy and considers their mechanisms of formation and toxicological implications.

MATERIALS AND METHODS

Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75.5 MHz, respectively, for solutions in 5 mm NMR tubes with a Bruker WM-300 spectrometer. The primary chemical shift references were tetramethylsilane for samples in organic solvents and TTP for aqueous solutions. The ^{1}H multiplicity, i.e. the number of ^{1}Hs directly attached to an observed ^{13}C, was determined by the DEPT pulse sequence [16]. 2-D NMR spectra [17] were recorded with a recycle time of 1.2 sec over a ^{13}C spectral width of 6400–9000 Hz (depending on the sample) with collection of 2048 data points which were exponentially multiplied to introduce a 0.125 Hz line broadening prior to Fourier transformation. All 2-D spectra were obtained by acquiring 160 scans for each of 256 experiments over a ^{1}H spectral width of 1560–2100 Hz, and the second dimension was generated by multiplication by an unshifted sine bell function and zero-filling by a factor of 2 prior to Fourier transformation. Spectra are presented in the absolute value mode. The digital resolution in the ^{1}H dimension of the 2-D spectra of metabolite mixtures was 6.0 Hz (or 0.02 ppm).

Synthesis of [3-^{13}C]*DBCP.* The synthesis of [3-^{13}C]DBCP (Fig. 1) is reported in detail because of considerable difficulties in obtaining a pure product. These problems included randomization of ^{13}C between C-3 and C-1 and formation of bromodichloropropanes and of high-boiling impurities that appeared to be halogenated ethers. Pentane was dried over metallic sodium, diethyl ether was freshly distilled from lithium aluminum hydride, and carbon tetrachloride and bromine were distilled from phosphorus pentoxide. Tetravinyltin was prepared from tin(IV) chloride and vinylmagnesium bromide in tetrahydrofuran [18]. All procedures involving vinyllithium were conducted under an argon atmosphere.

n-Butyllithium (48 mmol) in hexane (19.5 ml of 2.5 M solution) was added over 20 min with stirring to tetravinyltin (48 mmol) in pentane (50 ml). A white precipitate formed immediately, and the suspension was allowed to stand undisturbed for 4 hr, after which the solid vinyllithium was isolated by pressure filtration on a sintered glass disc, washed extensively with pentane, and dried with a stream of argon. The solid was dissolved in 30 ml of ether and transferred to a 3-necked flask fitted with a water-cooled reflux condenser. Dry [^{13}C]paraformaldehyde (16 mmol; 99 atom% ^{13}C, Cambridge Isotopes, Woburn, MA) was added under positive argon pressure, and the mixture came to spontaneous reflux, which was maintained with a heating mantle for 18 hr. After cooling to 20°, 0.9 ml of water was added in 0.1-ml portions and the suspension was filtered, washing the solids with ether (2 × 5 ml). The combined organic fractions were dried (0.5 g magnesium sulfate) and carefully distilled through a 12.5 cm Vigreaux column until the distillate temperature reached 35°. The residue was then subjected to trap-to-trap distillation at 1 mm to give 7.28 mmol of [1-^{13}C]allyl alcohol (determined from the weight of distillate and the ratio of allyl alcohol to diethyl ether based on integration of the ^{1}H NMR spectrum).

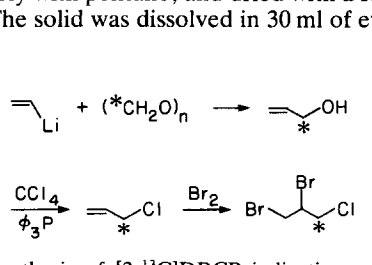

Fig. 1. Synthesis of [3-^{13}C]DBCP indicating position of labeling (*).

The [1-^{13}C]allyl alcohol/ether mixture was diluted with 7 ml of carbon tetrachloride, triphenylphosphine (14 mmol) was added, and the solution was heated at 45° in a closed system (allyl chloride boils at 46°). After 15 hr the resulting thick black sludge was subjected to trap-to-trap distillation (1 mm). Bromine (7.3 mmol) in carbon tetrachloride (1 ml) was added to the distillate, followed after 1 hr by treating the solution with 1.5 ml of 0.5 M sodium thiosulfate and separating the phases. The aqueous phase was washed with carbon tetrachloride (2 × 2 ml), the combined organic layers were dried (magnesium sulfate), and the carbon tetrachloride was distilled by gradually lowering the pressure to 6 mm. The [3-^{13}C]DBCP was isolated by short-path distillation at 1 mm (yield 1.41 g or 37% based on paraformaldehyde). ^{13}C NMR ($CDCl_3$); 33.4 ppm (C-1), 46.5 ppm (C-3), 48.7 ppm (d, J = 38 Hz, C-2). The purity of the [3-^{13}C]DBCP was ~98% with two minor signals at 47.8 and 48.2 ppm (1.1 to 1.2% each relative to the area of C-3 determined without ^{1}H nuclear Overhauser effects using a recycle time of 33.5 sec). The relative areas of C-1:C-2:C-3 of DBCP were 1.1:0.9:100. The identity of the product was confirmed by GC-MS.

Synthesis of GSH conjugates. A solution of GSH (5 mmol) in 40 ml of 50% aqueous methanol (adjusted to pH 8.5–9.0 with ammonium hydroxide) was treated with the alkylating agent (6 mmol) and allowed to react at 20° for 2.5 to 4.5 hr until no residual GSH was evident on treating a portion of the reaction mixture with 5,5′-dithiobis(2-nitrobenzoic acid). The conjugates prepared were *S*-(3-chloro-2-hydroxypropyl)GSH, *S*-(3-bromo-2-hydroxypropyl)GSH, *S*-(2,3-dihydroxypropyl)GSH and the *E*- and *Z*-isomers of *S*-[3-(1-chloropropenyl)]-GSH from the 3-chloro-, 3-bromo- and 3-hydroxy derivatives of 1,2-epoxypropane and from *E*/*Z*-1,3-dichloropropene (approximately 1:1 isomer ratio) respectively. On completion of the reactions, the solutions were extracted with chloroform (3 × 20 ml) and neutralized with hydrochloric acid; the methanol was removed at 20° *in vacuo* and then freeze-dried. Repeated attempts to crystallize the *S*-substituted-propyl derivatives failed. They were therefore characterized by ^{1}H and ^{13}C NMR and fast-atom bombardment MS as mixtures with contaminating ammonium chloride (Table 1). Small quantities

Table 1. ^{1}H and ^{13}C NMR and fast atom bombardment MS data for *S*-substituted GSH conjugates

Moiety			Chemical shift (ppm) in neutral D_2O						[M + H]§
			^{13}C*†			^{1}H†‡			
A	B	C	A	B	C	A	B	C	(*m/z*)
GS—	CH_2—	$CHOH$—CH_2Cl	38.1 38.0	72.7 72.5	50.6	2.81 (m)	4.06 (m)	3.78 3.70 (m)	400
GS—	CH_2—	$CHOH$—CH_2Br	39.1 39.0	72.2 72.1	40.2 40.1	2.82 (m)	4.02 (m)	3.65 3.58 (m)	444
GS—	CH_2—	$CHOH$—CH_2OH	37.8	73.8 73.5	67.2	2.88 2.76 (m)	3.94 (m)	3.76 3.66 (m)	382
GS—	CH_2—	(*E*) CH=$CHCl$	34.1	132.2	122.9	3.24 (d) ($J_{B\text{-}C}$ = 13.2 Hz)	5.98 (m)	6.29 (d)	382
GS—	CH_2—	(*Z*) CH=$CHCl$	30.1	130.3	123.7	3.41 (m) ($J_{B\text{-}C}$ = 7.1 Hz)	5.98 (m)	6.34 (d)	

* ^{13}C chemical shifts of carbons in the glutathionyl residue were similar in all conjugates. Typical values for *S*-(3-bromo-2-hydroxypropyl)GSH were: 178.7, 177.4, 176.5 and 174.4 ppm for the carboxyl and carboxamide carbons; 56.8, 28.9, and 34.1 ppm for C-2, C-3 and C-4, respectively, of the glutamyl residue; 55.9 and 36.3 ppm for C-2 and C-3, respectively, of the cysteinyl residue; and 46.1 ppm for C-2 of the glycyl residue.

† The first three conjugates exist as diastereotopic pairs because of the chiral center at the "B" position and, therefore, two ^{13}C signals were observed for this chiral carbon, and occasionally for the two flanking carbons. Certain of the diastereotopic ^{1}Hs also showed slight chemical shift differences, leading to complicated multiplets.

‡ Typical ^{1}H chemical shifts for the glutathionyl portion of *S*-(3-bromo-2-hydroxypropyl)GSH were: 3.80 (*t*, J = 6.3 Hz), 2.18 (m) and 2.56 ppm (m) for ^{1}Hs on C-2, C-3 and C-4, respectively, of the glutamyl residue; 4.61 (m) and 3.14 (dd) and 2.94 ppm (m) for ^{1}Hs on C-2 and C-3, respectively, of the cysteinyl residue; and 3.82 (d) and 3.75 (d) ppm (J = 17.2 Hz) for ^{1}Hs on the glycyl residue. All ^{1}H resonances (or groups of resonances when overlap occurred) gave appropriate integration.

§ Determined from a thioglycerol matrix with a Kratos MS-50 magnetic sector spectrometer in the positive ion mode with xenon as the reactant gas in the Department of Chemistry, University of California, Berkeley. Suitable isotope clusters were observed for the three compounds with bromo or chloro substituents.

(approximately 5 mg) of the *E* and *Z* isomers of *S*-[3-(1-chloropropenyl)]GSH were isolated by reverse phase HPLC (10 × 250 mm octadecylsilane column) by isocratic elution with water (pH 2.2 with hydrochloric acid) containing 16% acetonitrile. Complete ^{1}H and ^{13}C NMR assignments on all conjugates were carried out using appropriate ^{1}H-decoupling experiments, the ^{13}C DEPT pulse sequence, and 2-D NMR. In assigning the *E* and *Z* chloropropenyl derivatives, it is assumed that the *E* isomer has the larger olefinic ^{1}H-^{1}H coupling constant.

Metabolism of DBCP in rats. Male Sprague–Dawley rats (280–320 g from Charles River Laboratories Inc., Wilmington, MA) were anesthetized with sodium pentobarbital (70–85 mg/kg, i.p.) and the bile ducts were cannulated with the cannulas exiting the tail [19]. They were maintained individually in plastic metabolism cages with free lateral movement and access to food and water and were allowed to recover for 20–24 hr after surgery before administration of the test chemicals. Bile flow was 0.4 to 1.0 ml/hr.

Animals received i.p. doses of DBCP at 81 mg/kg (340 μmol/kg) in DMSO-d_6 (1 ml/kg) or DMSO-d_6 alone. The DBCP was either 3-^{13}C-labeled or non-enriched (a sample of analytical grade DBCP from the Shell Development Co., Modesto, CA); the latter material is referred to simply as DBCP. Both DBCP solutions contained a tracer amount (8 × 10^6 dpm per dose) of [U-^{14}C]DBCP (23.6 mCi/mmol, 97% radiochemically pure, a gift of the Shell Development Co.). In interpreting the results it is important to note that the ^{14}C-label is present in all three positions of the DBCP molecule, whereas only the 3-position is enriched with ^{13}C and that metabolism to $^{14}CO_2$ may occur in different amounts from each DBCP-derived carbon.

The DBCP administration route (i.p.) and high dose were selected to maximize the concentration of metabolites in the bile and urine since, for example, the NMR sensitivity is theoretically increased 16-fold at 80 versus 20 mg/kg. The dose chosen was that reported to cause only moderate hepatotoxicity when given s.c. in corn oil [4]. DMSO-d_6 was used as the carrier vehicle because it minimizes interfering ^{13}C NMR signals in three ways. First, the solvent resonance is split into a septet by the ^{2}Hs and the intensity is further reduced by the poor sensitivity of

this carbon resonance due to the long relaxation time characteristic of ^{2}H-substituted carbons. Second, since this carbon contains no Hs, it disappears completely in the DEPT spectra, thereby allowing detection of any ^{1}H-containing metabolite signals that might otherwise be masked by the solvent multiplet. Finally, the metabolite profile of this solvent is very simple, i.e. dimethylsulfone-d_6 (septet at 44.4 ppm) is the only expected metabolite [20], but it is not observed probably because the same factors that reduce the DMSO-d_6 signal render this metabolite undetectable.

Urine and bile, collected for 48 hr after dosing or until the animals died, were stored frozen. Bile was sampled every hour for the first 6 hr, then at 24 hr and at death. Urine was collected at 6 and 24 hr and at death. Excretion of radiocarbon in urine and bile was determined by liquid scintillation counting. Samples were prepared for ^{13}C NMR by mixing 360 μl of centrifuged bile or urine with 40 μl of D_2O and 5 μl of acetone. ^{13}C NMR spectra were recorded with broad-band ^{1}H-decoupling and a spectral width of 18,500 Hz (245 ppm) over 16,384 data points which were Fourier transformed after exponential multiplication to introduce a 2 Hz line-broadening. The pulse angle was 40°, the repetition rate was 2.4 sec, and the sample temperature was 25°. Acetone was used as the internal chemical shift reference (assigned 33.07 ppm, obtained from a sample consisting of 5 μl of acetone in 400 μl D_2O to which was added TTP).

Concentrated bile and urine samples were used to achieve the sensitivity required for 2-D NMR. The pooled 1-hr bile samples from the three DBCP-treated rats (3 ml) and those from the three [3-^{13}C]DBCP-treated rats (3 ml) were each extracted with ethyl acetate and then freeze-dried. The urine fractions from the first 24 hr from DBCP-treated rats (15 ml) and [3-^{13}C]DBCP-treated rats (9 ml) were each pooled and brought to 20 ml with water, treated with urease (40 mg) for 3 hr at 20°, and freeze-dried. Each residue from bile dissolved easily in 350 μl of D_2O and was used directly for 2-D NMR. Each residue from urine, with a higher content of inorganic salts, was extracted successively with ethyl acetate (10 ml) and methanol (10 ml) by sonication, and the methanol extract (containing 75% of the urinary radiocarbon) was reduced to dryness *in vacuo* prior to dissolution in 350 μl of D_2O for 2-D NMR.

RESULTS

Excretion of [^{14}C]DBCP-derived radiocarbon. DBCP-derived radiocarbon appeared rapidly in the bile with the highest concentration during the first hour after treatment and a steady decline thereafter (Table 2). Little radiocarbon was excreted during the 24–48 hr post-treatment interval, but three of the five DBCP-treated animals died during this period. Ethyl acetate extracted 0.3 to 0.4% of the biliary radiocarbon (not further characterized) and <0.1% of the urinary radiocarbon.

^{13}C NMR spectroscopy of 0–1 hr bile. Spectra from bile of two rats given only DMSO-d_6 (control) exhibited several very weak signals in addition to the acetone resonances (the carbonyl resonance of acetone at 218.1 ppm is not shown) (Fig. 2). Four additional resonances were evident in the bile of three rats given DBCP and DMSO-d_6, and they were also present at the same relative intensity in the bile from rats given [^{13}C]DBCP and DMSO-d_6. These signals were associated with GSH conjugates whose concentration in bile was increased on administration of DBCP. Spectra of the bile of three [^{13}C]DBCP-treated rats revealed fifteen signals attributable to ^{13}C-enriched metabolites. All fifteen signals were present in each of the treated rats, and the relative signal intensities were very reproducible, e.g. B13 was always the most intense metabolite peak (Table 3). The signals designated as B3, B5, and B6 were broad, and each probably represented two closely-spaced carbons. Metabolites associated with the B_7 and B_{10} signals were no longer detected in the concentrated bile sample used for 2-D NMR, indicating their instability or volatility.

Table 2. Radiocarbon in the bile, urine and feces of rats administered [^{14}C]DBCP, i.p., at 81 mg/kg

Sample	Recovery of administered radiocarbon* (%)
Bile, hr	
0–1	8.5 ± 2.2
1–6	12.3 ± 2.8
6–24	15.2 ± 5.4
Subtotal	36.0 ± 9.4
Urine, hr	
0–6	10.2 ± 2.6
6–24	10.4 ± 2.6
Subtotal	20.6 ± 8.1
Feces, 0–24 hr	<0.05
Total, 0–24 hr	56.6 ± 8.1

* Each value is the mean ± SD of results with five [^{14}C]DBCP-treated rats, three receiving DBCP and two receiving [3-^{13}C]DBCP. A third rat dosed with [^{13}C]DBCP eliminated 7.2% of the ^{14}C in the 0–6 hr urine and 7.0% in the 1 hr bile and died 8–22 hr after treatment. Radiocarbon in the 24–48 hr bile was 3.5 and 0.9% of the dose for two rats surviving 48 hr and 2.3, 3.5 and 5.7% of the dose for three rats dying within the 24–48 hr post-treatment interval. Radiocarbon in the 24–48 hr urine ranged from 0.1 to 0.9% of the dose, but the volume of urine was abnormally low and three of the five rats died during this period.

^{13}C NMR spectroscopy of 0–6 hr urine. Recognition of metabolites was complicated by the large number of signals detected in control urine and by differences in the volume of urine produced by DBCP-treated rats (1.9 ± 1.1 ml) versus controls (5.8 and 7.7 ml) (Fig. 3). Signals from endogenous substances were generally more intense (relative to acetone) in the more concentrated urine from the treated animals. None of the peaks identified as urinary metabolites were present in a 10-fold concentrated sample of control urine from this period, although signals from additional endogenous substances were detected (spectrum not shown). The

Table 3. ^{13}C-Enriched metabolites detected by NMR spectroscopy in the bile and urine of [3-^{13}C]DBCP-treated rats*

Bile					Urine				
	^{13}C			^{1}H		^{13}C			^{1}H
Metabolite signal	Chemical shift† (ppm)	Relative intensity‡ (%)	Multiplicity§	Chemical shift‖ (ppm)	Metabolite signal	Chemical shift† (ppm)	Relative intensity‡ (%)	Multiplicity§	Chemical shift‖ (ppm)
B1	30.8	10 ± 2	CH_2	2.73	U1	30.8	(7) (8)¶	CH_2	**
B2	31.0	14 ± 8	CH_2	2.86					
B3	36.8††	42 ± 5	CH_2	2.99	U3	37.0	19 ± 5	CH_2	3.29
				2.91					3.01
B4	37.6	38 ± 6	CH_2	2.88	U4	37.7	38 ± 1	CH_2	2.82
				2.75					
B5	39.0††	20 ± 5	CH_2	3.05					
B6	40.2††	17 ± 2	CH_2	2.97					
				2.80					
B7	42.6	21 ± 14	CH_2	**					
					U8	44.6	15 ± 6	CH_2	3.66
					U9	48.5	11 ± 2	CH_2	4.15
B10	50.2	9 ± 1		**					
B11	50.6	32 ± 1	CH_2	3.84‡‡	U11	50.7	36 ± 4	CH_2	3.85‡‡
B12	63.1	14 ± 4	CH_2	3.76					
B13	64.2	100	CH_2	3.84	U13	64.2	100	CH_2	3.85
B14	65.6	28 ± 1	CH_2	3.84	U14	65.4	19 ± 1	CH_2	3.86
B15	67.1	22 ± 1	CH_2	3.79	U15	67.1	16 ± 3	CH_2	3.74
				3.68					
					U16	81.0	8 ± 2	CH_1	**
B17	122.9	8 ± 3	§§	6.40‡‡	U17	122.7	8 ± 2	CH_1	6.36‡‡
B18	123.7	12 ± 6	CH_1	6.42‡‡	U18	123.4	12 ± 3	CH_1	6.40‡‡

* Compounds not observed as biliary or urinary metabolites and their relevant chemical shifts (ppm) *in water* were: DBCP, 49.8 (C-3); 2,3-dibromopropanol, 66.1 (C-1); 2-bromo-2-propenol, 69.3 (C-1) and 120.3 (C-3); sodium 2-bromo-2-propenoate, 172.0 (C-1) and 128.6 (C-3); sodium 3-chlorolactate, 50.6 (C-3) with attached ^{1}Hs at 3.96; sodium chloroacetate, 46.7 (C-2); sodium glycolate, 64.1 (C-2) with attached ^{1}Hs at 4.04; sodium glyoxylate hydrate, 90.8 (C-2); trisodium citrate, 48.6 (CH_2) with attached ^{1}Hs at 2.77 and 2.60; and trisodium isocitrate, 40.5 (CH_2) with attached ^{1}Hs at 2.62 and 2.52.

† See Figs. 2 and 3 for typical ^{13}C NMR spectra of metabolites in bile and urine respectively.

‡ Peak heights relative to B13 or U13, the most intense metabolite signals observed. Values are means ± SD, N = 3.

§ ^{1}H multiplicity was determined from DEPT spectra.

‖ Chemical shifts of ^{1}H atoms attached to ^{13}C atoms were determined on concentrated bile and urine samples.

¶ Detected in only two of three urine samples.

** Not detected in concentrated samples used for 2-D NMR.

†† Broad signals probably representing two closely-spaced resonances.

‡‡ Values of ^{1}H chemical shifts of metabolite standards reported in Table 1 are obtained from high resolution one-dimensional NMR spectra. Slight discrepancies between the values reported here and those of Table 1 were due to the poorer digital resolution and distortion of the multiplet shape in the 2-D spectra. However, cross-peaks observed in 2-D spectra of synthetic standards matched the cross-peaks of metabolites listed here.

§§ DEPT experiment gave equivocal results but detection of an attached proton in the 2-D NMR spectrum and the value of the ^{1}H and ^{13}C chemical shifts strongly suggest that this is a CH_1.

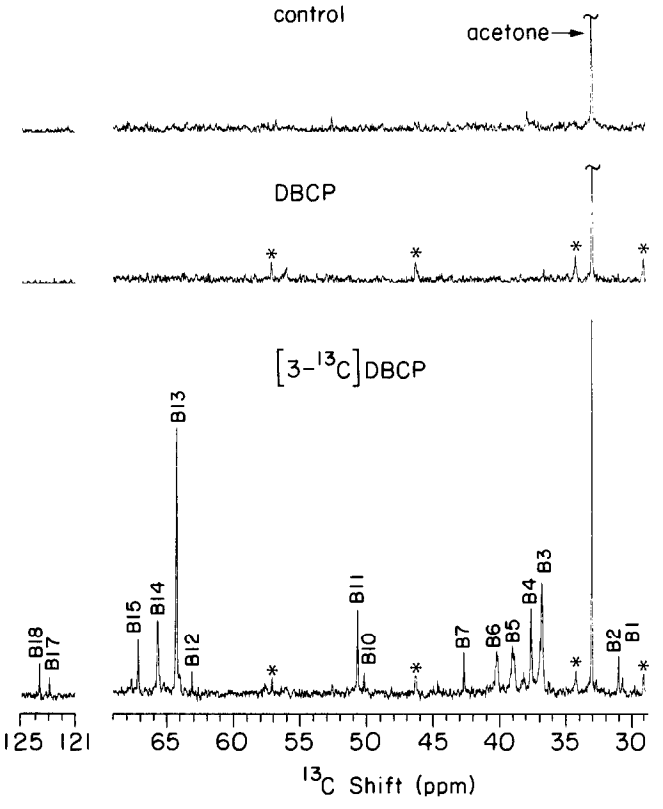

Fig. 2. Partial ^{13}C NMR spectra of bile from control rats and those given DBCP or [3-^{13}C]DBCP. Asterisks designate signals associated with GSH conjugates whose concentration in bile was increased on administration of DBCP, i.e. C-2, C-3 and C-4 of the glutamyl moiety and C-2 of the glycyl moiety (see first footnote of Table 1). Signals derived from C-3 of [3-^{13}C]DBCP were B1–B7, B10–B15, B17 and B18 in order of increasing chemical shifts. Acetone was added as a chemical shift reference.

presence of DMSO-d_6 in all urinary samples was indicated by the characteristic septet at 40.7 ppm. Most of the endogenous signals were not identified, but an intense resonance at 165.5 ppm (not shown) was due to urea, and the CH_2 and CH_3 carbons of creatine were detected at 56.6 and 39.7 ppm respectively. Twelve signals were attributable to ^{13}C-enriched metabolites, present in a highly reproducible pattern, except for U1, which was detected in only two of the three animals.

Nature and identity of biliary and urinary metabolites. A 2-D NMR spectrum of the concentrated bile sample from the [3-^{13}C]DBCP-treated rats is shown as a contour plot with two representative cross sections in Fig. 4. Chemical shifts of the protons directly attached to the ^{13}C-enriched carbons of biliary and urinary metabolites are summarized in Table 3. DEPT spectra indicated that most of the metabolite signals detected were from CH_2 carbons, except for two biliary and three urinary metabolites which contained ^{13}C-labeled CH_1 carbons. No DBCP was detected nor were any metabolite signals in the region corresponding to carboxaldehyde, carboxylic acid or carboxamide functionalities (160–200 ppm). Several potential metabolites not observed by ^{13}C NMR are listed in the first footnote to Table 3.

Two signals from ^{13}C-enriched metabolites appeared in the olefinic region in both bile (122.9 and 123.7 ppm) and urine (122.7 and 123.4 ppm). All four signals were due to CH_1 units, and their directly-attached ^1Hs had chemical shifts of 6.36–6.42 ppm. The ^{13}C chemical shifts of biliary metabolites B_{17} and B_{18} were identical to those of the chloro-substituted carbon in synthetic *E* (122.9 ppm) and *Z* (123.7 ppm) *S*-[3-(1-chloropropenyl)]GSH, respectively, and the ^1H chemical shifts of the directly-attached ^1Hs matched as well. The very similar ^{13}C and ^1H chemical shifts of the corresponding urinary metabolites (U_{17} and U_{18}) indicated that they were also *S*-[*E*- or *Z*-3-(1-chloropropenyl)] derivatives. The slight differences in ^{13}C shifts between the biliary and urinary metabolites probably reflect different *S*-substituents, e.g. glutathionyl versus *N*-acetylcysteinyl.

The biliary metabolite signals at 67.1 ppm (B_{15}) and 37.6 ppm (B_4) were assigned to *S*-(2,3-dihydroxypropyl)GSH labeled at C-3 and C-1 of the 2,3-dihydroxypropyl moiety respectively. The chemical shifts for both ^{13}Cs and the attached ^1Hs matched exactly those of the synthetic compounds. Additionally, two ^1Hs on each labeled carbon showed sufficient non-equivalence to appear as separate signals in the 2-D NMR spectrum (Fig. 4), as did the corresponding ^1Hs of the synthetic standard. Urinary metabolite signals U_{15} and U_4 also cor-

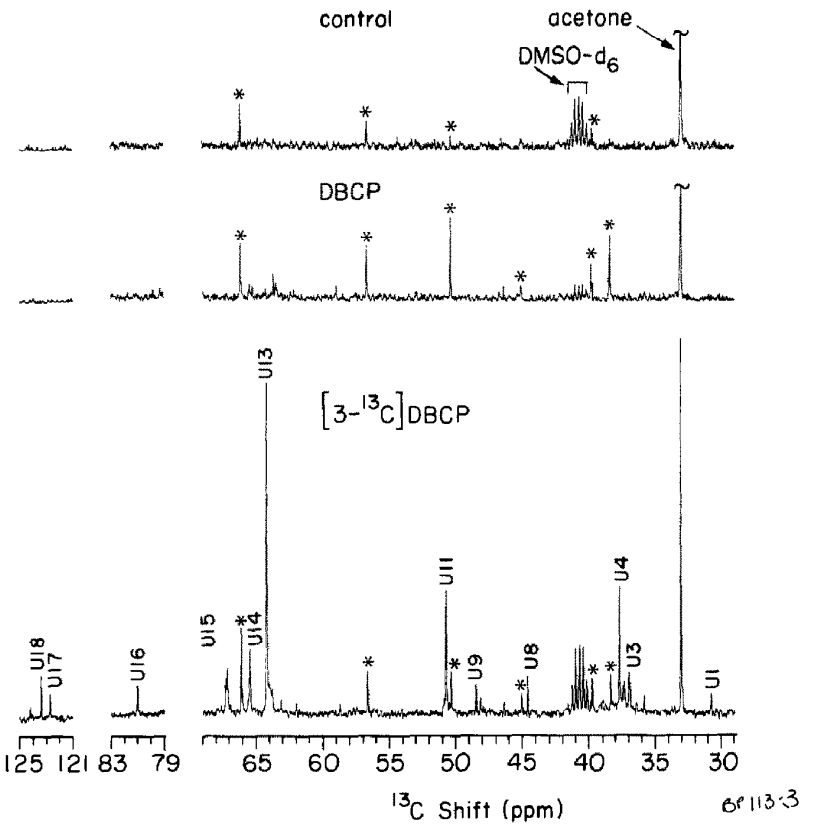

Fig. 3. Partial ^{13}C NMR spectra of urine from control rats and those given DBCP or [3-^{13}C]DBCP. Asterisks designate endogenous substances in control urine. Signals derived from C-3 of [3-^{13}C]DBCP are designated U1, U3, U4, U8, U9, U11, and U13–U18 in order of increasing chemical shifts. Acetone was added as a chemical shift reference.

responded to an *S*-(2,3-dihydroxypropyl) derivative labeled at C-3 and C-1, respectively, except that U_{15} gave only a single cross-peak in the 2-D NMR spectrum.

The biliary metabolite signal at 50.6 ppm (B_{11}) and its ^{1}H signal at 3.84 ppm corresponded to those of *S*-(3-chloro-2-hydroxypropyl)GSH labeled at C-3 of the chlorohydroxypropyl moiety. U_{11} was the equivalent urinary metabolite. The bromo analog, *S*-(3-bromo-2-hydroxypropyl)GSH, had an *S*-CH_2 signal corresponding to B_5 but the ^{1}H resonances detected in the 2-D spectrum of the metabolite did not match those of the standard.

Metabolite signals in the 63–66 ppm range (B_{12}, B_{13}, B_{14}, U_{13}, and U_{14}) were not individually assigned but their ^{13}C and ^{1}H chemical shifts and their ^{1}H multiplicities indicated that in these metabolites C_3 of DBCP is transformed into a primary alcohol (or conjugate thereof). B_{10} probably still contains a $^{13}CH_2Cl$ functionality, based solely on its ^{13}C chemical shift of 50.2 ppm. Signals in the 31–40 ppm range (B_1, B_2, B_3, B_5, B_6, U_1, and U_3) were attributable to compounds of the type $RS^{13}CH_2R'$, based on their ^{13}C and ^{1}H chemical shifts, but the identity of the R′ substituent is unknown. The nature of B_7, U_8, and U_9 is problematical with the available data. Their ^{13}C signals were too far downfield for *S*-substituted derivatives (except perhaps B_7 at 42.6 ppm) and too far upfield to retain the chlorine atom (except U_9 at 48.5 ppm). It is possible that these metabolites result from reaction of C-3 with a nitrogen-containing nucleophile. The urinary metabolite at 81.0 ppm (U_{16}) is also of unknown nature.

DISCUSSION

DBCP-derived radiocarbon was extensively excreted and appeared more rapidly in the bile following i.p. than oral administration (Table 4). A comparison of cannulated versus non-cannulated rats indicates that an appreciable fraction of the biliary metabolites was reabsorbed and excreted in the urine, perhaps after further alteration. The percent of the dose eliminated from the body, essentially all as metabolites, during the 0–24 hr period was not greatly different at doses of 11, 20, and 81 mg/kg, indicating that the capacity of the rat to metabolize DBCP is maintained even at the latter lethal dose.

Possible mechanisms for production of $^{14}CO_2$ include metabolism of DBCP either to a tricarboxylic acid cycle intermediate or to an α-keto acid such as 3-chloro- or 3-bromopyruvate which undergoes oxidative decarboxylation. However, the expected products of these pathways were not detected by ^{13}C NMR, i.e. citrate and isocitrate (the first two

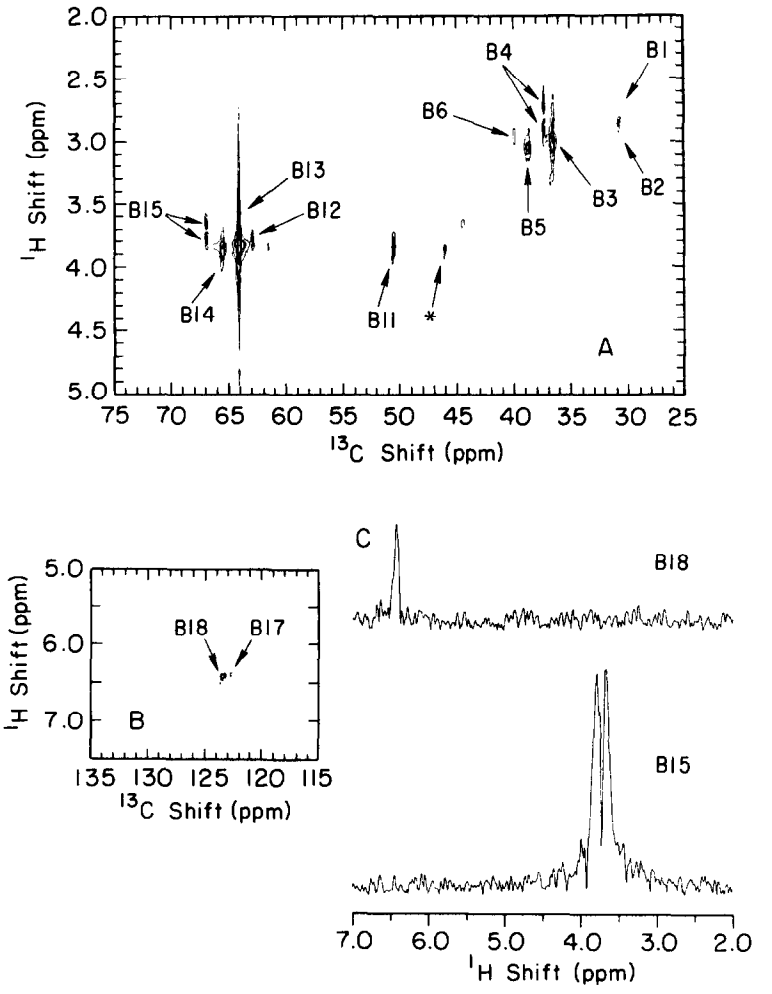

Fig. 4. 2-D NMR spectrum of biliary metabolites of [3-^{13}C]DBCP (panels A and B) and representative ^{1}H cross sections for B15 and B18 (panel C). Metabolites and an endogenous substance (*) are designated as in Fig. 2.

intermediates in the tricarboxylic acid cycle) and chloroacetate, glycolate, and glyoxylate (possible products of α-keto acid decarboxylation). Also not detected were 3-chloro- and 3-bromolactate (reduction products of α-keto acids) and oxalate, chemicals previously reported as DBCP metabolites [21].

The four identified metabolites of DBCP are sulfur-based conjugates, one of which is labeled in two positions. The NMR experiments identified the

Table 4. Effect of treatment protocol on radiocarbon in the bile and excreta of rats 24 hr after administration of [^{14}C]DBCP

Treatment protocol				Recovery of administered radiocarbon (%)				
Bile cannula	Administration route	Dose (mg/kg)	^{14}C Position	Bile	Urine	Feces	$^{14}CO_2$	Reference
Yes	i.p.	81	U	36*	21	<0.05		This study
Yes	oral	20	3	23†	19	0.2		11
No	oral	20	3		48	14	17	11‡
No	oral	11	U		50	14	16	13

* Cumulative percent excretion of 8.5, 15.1 and 20.8% in 1, 3 and 6 hr respectively.
† Cumulative percent excretion of 1.7 and 5.8% in 3 and 6 hr respectively.
‡ Extensive radiocarbon retention in tissues.

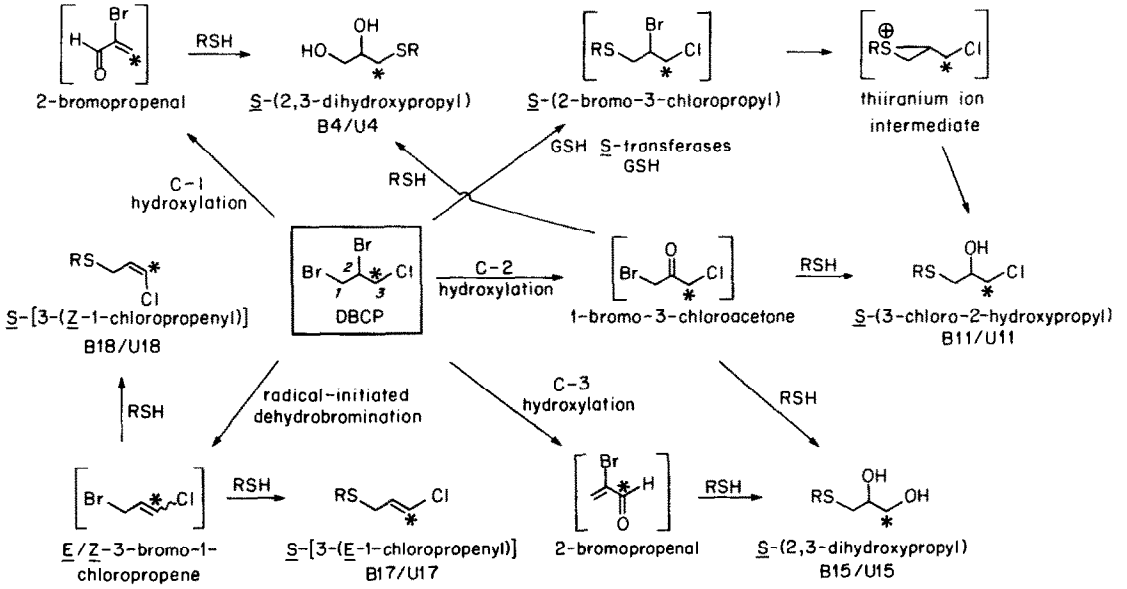

Fig. 5. Four *S*-conjugate metabolites of [3-^{13}C]DBCP and partial pathways for their formation by reaction with GSH or radical-initiated dehydrobromination or hydroxylation at C-1, C-2 or C-3 with subsequent reactions with GSH. Some of the reactions involved multiple steps as shown in Figs. 6 and 7. R refers to glutathionyl, cysteinylglycine or cysteinyl for the biliary metabolites and cysteinyl or *N*-acetylcysteinyl for the urinary metabolites. Identified metabolites are designated as in Table 3.

nature of the DBCP-derived portion but not of the conjugating moiety. The conjugates undoubtedly are derived from GSH and are present as cysteinyl and predominantly *N*-acetylcysteinyl derivatives in urine and as GSH, cysteinylglycine or cysteinyl derivatives in bile, in the latter case by analogy with the normal presence in bile of GSH and its hydrolysis products [22]. The metabolites identified were *S*-(3-chloro-2-hydroxypropyl) and *S*-(2,3-dihydroxypropyl) (labeled at C-1 and C-3) derivatives and *S*-(*E*- and *Z*-1-chloropropenyl) derivatives. *S*-(3-Chloro-2-hydroxypropyl)- and *S*-(2,3-dihydroxypropyl)-*N*-acetylcysteine are known urinary metabolites of DBCP in rats [13, 21]. Three general mechanisms of formation of these GSH-derived conjugates are shown in Fig. 5 and discussed below, i.e. direct GSH conjugation, radical processes, and oxidative pathways.

Reaction of GSH at C-1 of DBCP yields a highly-reactive sulfur mustard which hydrolyzes via a thiiranium ion intermediate to give *S*-(3-chloro-2-hydroxypropyl)GSH (Fig. 5). Analogous attack at C-3 would give *S*-(3-bromo-2-hydroxypropyl)GSH, which was not detected. Reaction of GSH at C-2 of DBCP would probably yield the same products because of the tendency of the intermediate thiiranium ions to form primary thioethers [23]. Direct GSH conjugation does not appear to be a major pathway since DBCP and GSH did not react at a detectable rate under physiological conditions (unpublished observation) and DBCP is a very poor substrate for rat GSH *S*-transferases [24].

Radical processes are invoked to explain the formation of both the *E*- and *Z*-isomers of *S*-[3-(1-chloropropenyl)]GSH which is rationalized as occurring by reaction of GSH (either directly or enzyme-catalyzed) with *E*- or *Z*-3-bromo-1-chloropropene (Fig. 5). These allylic bromides are formed from DBCP by bromine radical abstraction at C-2, most likely catalyzed by a reduced hemoprotein. Expulsion of a hydrogen radical (Hs on the chlorinated carbon would be the most labile) gives the requisite alkene.

Oxidation of DBCP at C-3 to produce a *gem*-chlorohydrin would give 2,3-dibromopropanal which rapidly dehydrobrominates under physiological conditions [25] and oxidation at C-1 is expected to give an analogous series of reactions (Fig. 6). Further evidence for 2-bromopropenal as an intermediary metabolite of DBCP comes from detecting trace amounts of 2-bromopropenoic acid in incubates of DBCP with liver microsomes and NADPH and in the urine of DBCP-treated rats [26]. The 2-bromopropenal formed reacts rapidly with GSH by Michael addition to give (via thiiranium ion-mediated hydrolysis) *S*-(2-hydroxy-3-oxopropyl)-GSH (unpublished observation, exists as the hydrate in aqueous solution at pH 7) which on reduction of the aldehyde functionality would form *S*-(2,3-dihydroxypropyl)GSH (Fig. 7). Oxidation of [3-^{13}C]DBCP at C-1 and C-3 would, therefore, yield *S*-([1-^{13}C]- and [3-^{13}C]2,3-dihydroxypropyl)GSH respectively. By analogy, the major metabolite of 2-propenal in the rat is *S*-(3-hydroxypropyl)-*N*-acetylcysteine [27], presumably formed by Michael addition of GSH and subsequent reduction of the aldehyde functionality.

Oxidation of DBCP at C-2 would give a *gem*-bromohydrin which dehydrobrominates to 1-bromo-3-chloroacetone (Fig. 6), a bifunctional alkylating agent which can readily react with a thiol, as shown in Fig. 7. These *S*-(3-substituted-2-oxopropyl)GSH

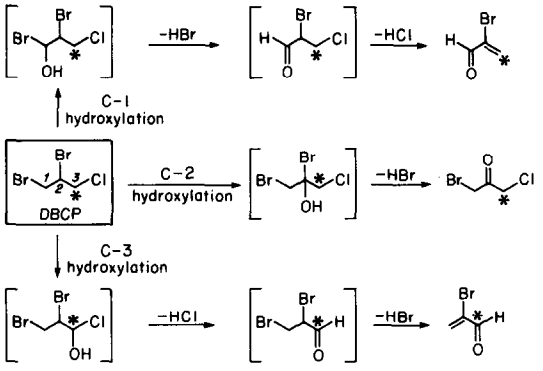

Fig. 6. Possible conversion of [3-^{13}C]DBCP to [3- or 1-^{13}C]2-bromopropenal on C-1 or C-3 hydroxylation and to [3-^{13}C]1-bromo-3-chloroacetone on C-2 hydroxylation.

derivatives have not been detected but the unidentified metabolites may include compounds of this type. Furthermore, reduction of the ketone functionality of *S*-(3-chloro-2-oxopropyl)GSH and *S*-(3-hydroxy-2-oxopropyl)GSH provides alternative pathways for the formation of the 3-chloro-2-hydroxypropyl and 2,3-dihydroxypropyl derivatives respectively. On an analogous basis, studies on the metabolism of 1,2-dibromopropane show extensive oxidation at C-2 and suggest that the ketone functionality of *S*-(2-oxopropyl)GSH is reduced *in vivo* in the rat [28].

Several bioactivation products formed by direct conjugation with GSH or by oxidation at any one of the carbon atoms may contribute to the diverse toxicological effects of DBCP. GSH plays a major role in DBCP metabolism, undergoing pronounced depletion in liver with high DBCP doses and serving as the conjugating moiety in many of the metabolites.

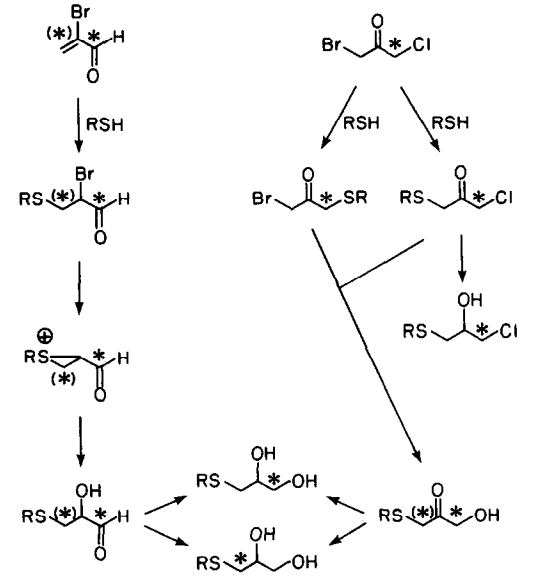

Fig. 7. 2-Bromopropenal and 1-bromo-3-chloroacetone as possible intermediates in formation of *S*-(2,3-dihydroxypropyl)- and *S*-(3-chloro-2-hydroxypropyl)GSH derivatives.

Reactive GSH conjugates (or their cysteinylglycine or cysteinyl derivatives) often mediate the nephrotoxicity of haloalkanes and haloalkenes [29]. Direct reaction of DBCP with GSH to yield reactive sulfur mustards appears to be a minor metabolic pathway as discussed above and, therefore, is probably not a significant factor in either acute toxicity or genotoxicity. Thus, in marked contrast to 1,2-dibromoethane [24], the bacterial mutagenicity [10] and *in vitro* DNA binding of DBCP [24] are not mediated by GSH and GSH *S*-transferases.

Oxidation of DBCP at C-1 or C-3, yielding 2-bromopropenal, is a pathway that may account for both the *in vivo* GSH depletion and the bacterial mutagenicity. 2-Bromopropenal is a direct-acting and extremely potent mutagen (~1000 revertants/nmol in the Ames' *Salmonella* strain TA 100 assay) [25]. The requirement for microsomal oxidation in bacterial mutagenicity is consistent with 2-bromopropenal being the ultimate mutagen. The GSH conjugate of 2-bromopropenal, i.e. *S*-(2-bromo-3-oxopropyl)GSH, may accumulate in the kidney yielding a high concentration of reactive thiiranium ions leading to the nephrotoxicity.

1-Bromo-3-chloroacetone potentially formed on oxidation of DBCP at C-2 may contribute to its rapid GSH depletion and ultimately yield *S*-(3-halo-2-oxopropyl)GSHs which, as α-haloketones and GSH conjugates, would be powerful alkylating agents, potential nephrotoxins, and candidate testicular toxins. 1-Bromo-3-chloroacetone may also act directly as a genotoxin since the dichloro analog is a potent mutagen in bacteria (e.g. ~14 revertants/nmol, *Salmonella* strain TA 100) [30] and in the sister chromatid exchange test [31].

In summary, this investigation used highly specific ^{13}C and ^{1}H NMR techniques to identify a series of very polar metabolites of DBCP in the bile and urine without separation or potential degradation. It will serve as a guide in future studies on the unidentified metabolites and in elucidating the mechanisms by which DBCP causes its diverse toxic effects.

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