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THE METABOLISM OF ETHYLENE DIBROMIDE IN THE RAT

THE ENZYMIC REACTION WITH GLUTATHIONE *IN VITRO* AND *IN VIVO**

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Abstract—The reaction of glutathione and ethylene dibromide (EDB) was studied *in vitro* in the presence of rat liver homogenate. It was found that the release of two bromide ions from EDB required stoichiometric amounts of GSH. S-(β-hydroxyethyl) glutathione has been identified as the major product formed *in vitro*. S-S′ ethylene bis glutathione was formed in traces. The above two compounds and the sulphoxides of S-(β-hydroxyethyl) glutathione were identified also in the liver of rats treated with labeled EDB. S-(β-hydroxyethyl) mercapturic acid was identified in the kidney.

ETHYLENE dibromide (EDB) when administered orally to rats was found to depress the level of thiol groups.[1] It was also found to form mercapturic acid derivatives *N*-acetyl-S-(β-hydroxyethyl)-L-cysteine in urine.[2] These observations were in accordance with several types of compounds which form mercapturic acid derivatives.[3,4] Glutathione derivatives were assumed to be intermediate products in the metabolism of halogeno-alkanes.[5] Several glutathione S-transferases have been described which are responsible for combining glutathione with mercapturic acid precursors.[6–9] To provide evidence for this mechanism in EDB poisoning, we studied the enzymic reaction of glutathione with EDB, and the derivatives formed *in vitro*. The metabolites formed *in vivo*, following EDB administration to rats, were also identified.

MATERIALS

EDTA, EDB, ethylene bromohydrin (EBH) and triethyl amine were from BDH; GSH, GSSG, NADPH, *N*-ethyl maleimide (NEM) from Sigma Chemical Co.; and DTNB, from the Aldrich Chemical Co.

S-(β-hydroxyethyl) glutathione ($GSCH_2CH_2OH$) was prepared by the reaction of GSH and EBH by a method similar to that described by Zilka and Weinstein[10] for the preparation of S-(β-hydroxypropyl)-L-cysteine. The product of the reaction was dried by distillation *in vacuo* at 0°, dissolved in 90 per cent alcohol and precipitated with ether. The product was recrystallized from alcohol–ether 20:1. The crystals passed to oil phase at 128° and to liquid phase at 162°.

S-S′ ethylene-bis-glutathione ($GSCH_2CH_2SG$) was prepared by reacting 1 g (0·003 M) GSH with 0·67 g (0·003 M) EDB in a solution of 8 ml alcohol 75 per cent

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in the presence of 0·01 M triethylamine at 0°. The reaction mixture was dried *in vacuo* and the compound recrystallized from alcohol 75 per cent, m.p. 180°. The end of the reactions was established by the complete disappearance of free —SH groups.[11]

S-(β-hydroxyethyl) mercapturic acid was prepared as described in a previous paper.[2]

METHODS

Enzyme preparation

Female white rats weighing about 200 g were used throughout the experiment. Livers of rats, immediately after killing, were homogenized with a Teflon homogenizer in ice cold phosphate buffer 0·1 M, pH 7·4 at a ratio of 1:5. The homogenate was centrifuged for 15 min at 3000 *g*, after which the supernatant was centrifuged for 60 min at 100,000 *g* in a Spinco Model ultra centrifuge. The same procedure was followed to obtain the kidney homogenate. The supernatants were used immediately for activity determinations. Protein was determined by micro Kjeldahl digestion and the modified Nessler's reagent.[12] One ml of liver supernatant contained 3·5 mg N; that of kidney contained 2·5 mg of N.

Enzyme assays. The assays were carried out in the phosphate buffer described above with 2–4 mM GSH; 2–4 mM EDTA; 3–4 mM EDB added in 0·05 ml acetone solution and 0·5 ml of an enzyme preparation (equivalent to 150 mg tissue). The total volume, 6·5 ml, was incubated in a shaking water bath at 37°. The rate of the enzyme reaction was determined by:

(a) Disappearance of thiol groups: A slightly modified Ellman[11] procedure was used with the reagent S,S'-dithiobis-(2-nitrobenzoic acid). Duplicates of 0·1 ml samples were withdrawn from the reaction mixture every 10 min. The samples were added to tubes containing 3 ml of 0·3 M Na_2HPO_4; 0·5 ml of DTNB solution (0·04 per cent in 0·3 M Na_2HPO_4, pH 7·7) was subsequently added. The tubes were mixed quickly. The yellow color of mixed disulphide was read at 412 mμ.

(b) Production of Br ions: Bromine was determined by the method of Hunter[13] and by the neutron activation method of Shenberg *et al.*[14] The samples were counted on a Lithium Drifted Silicon Detector (25 mm^2 area; Nuclear Equipment Corporation). The detector was coupled through a low noise preamplifier and an Ortec Model 410 Linear Amplifier to a 400-channel analyzer. Under optimum conditions, the resolution was 310–330 eV. Cobalt-57 (6·8 keV X-ray and 14·4 keV gamma ray) was used for the energy calibration. This method was chosen because bromine can be determined in μg in the presence of interfering salts. Samples of the reaction mixture (0·1 ml) were withdrawn at fixed intervals of time during the reaction and absorbed on Whatman No. 1 filter paper discs of 2·5-cm diameter. The unchanged volatile substrate, water and ethanol were evaporated from the disc by blowing hot air on its surface until dry. The corresponding two blanks consisted of: (1) boiled enzyme + EDB, and (2) EDB + GSH. Traces of non-volatile bromine were found in these blanks and were subtracted from the results.

(c) Production of non-volatile radioactive glutathione derivatives; [U-^{14}C] EDB with a sp. act. of 6·5 mc/m-mole. The radioactive compound was diluted with non-radioactive EDB and stored in the refrigerator. This solution was diluted with acetone before use, to contain 1 μc in aliquots of 0·05 ml. Sampling was carried out as described above, in method (b). The filter paper discs were placed in scintillation counter vials

and after rapid drying in a fume cupboard, as above, 8 ml of toluene scintillation solution was added to each vial.

The radioactivity of the sample was measured in a Packard TriCarb scintillator. The efficiency of counting was 70–75 per cent and was not affected by traces of insoluble salts or denatured protein in the mixture.

Oxidized glutathione. This was determined by the enzymic method[15] using the SH reagents DTNB and NEM, to verify that no autoxidation or side reactions had occurred during incubation. This method determines nanograms of GSSG.

Isolation of the products of enzymic reaction in vitro. The products of the reaction in method (c) were prepared by permitting the reaction to run to completion within 1 hr. The reaction mixture was then boiled for 2 min to denature the protein, centrifuged for 15 min at 600° *g*, and the supernatant decanted and evaporated to dryness *in vacuo*. The residue was dissolved in 80% alcohol. Aliquots of this solution were chromatographed.

Isolation of metabolic product of EDB from liver and kidney. [U^{14}C] labeled EDB was diluted in soybean oil (1:50) and administered by stomach tube to rats starved for 16 hr prior to treatment. The dose containing 10 μc per animal was given in 0·12 g EDB/kg body wt. The animals were killed after 4 hr. The liver and the kidney were homogenized separately in 80% alcohol in 1:5 ratio and centrifuged at 6000 *g* for 20 min. The supernatants were dried *in vacuo* and redissolved in 80% alcohol. These solutions were used for separation and identification of the metabolites.

Chromatography

Radioactive samples were chromatographed on strips of 2·5-cm wide Whatman 1 MM paper in monodimensional chromatograms and scanned for radioactivity with a Packard Model 7200 Radiochromatogram Scanner. This method was also used to prepare relatively pure compounds from the tissue extract, by preparative chromatography on Whatman 3 MM filter paper. Descending mono- and two-dimensional chromatography were employed. The following solvents were used: (A) butan-1-ol–acetic acid–water (75:21:42 v/v). (B) phenol–water (4:1 v/v); (C) propan-1-ol–pyridine–water (1:1:1 v/v).

Thin layer chromatography employing cellulose and kieselgel as coating was used.[16] The solvents used were: (D) butan-1-ol–acetic acid–water (80:20:20 v/v); (E) butan-1-ol–acetone–diethylamine–water (10:10:2:5 v/v). Sulfur compounds were detected with platinic acid,[17] as modified by Barnsley.[18] Amino acids were detected by dipping the chromatograms in 0·2% (w/v) ninhydrin in acetone, to which pyridine, 2% (v/v), had been added.

RESULTS

The reaction between EDB and GSH was catalysed by an enzyme present in the supernatant of rat liver homogenate. The molar consumption of thiol groups and the production of two Br ions were found to be equivalent. This was determined by employing methods (a) and (b); the results obtained from three experiments are summarized in Fig. 1. The rate of GSH disappearance plotted against sampling time was a straight line during the initial 30 min of the reaction. The activity of the enzyme present in the supernatant of rat liver varied from rat to rat, but the ratio between thiol consumption and Br ion production was constant.

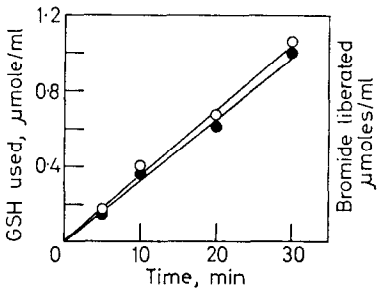

FIG. 1. Correlation between GSH consumption and bromine liberation. Reaction conducted at 37° in phosphate buffer 0·1 M, pH 7·4 for 30 min. Test solution contained: GSH (2mM); EDTA (4mM); EDB (3mM); and enzyme preparation 0·5 ml which is equivalent to 130 mg liver; in a total volume of 6·5 ml. ○, GSH disappearance; ●, bromine liberation.

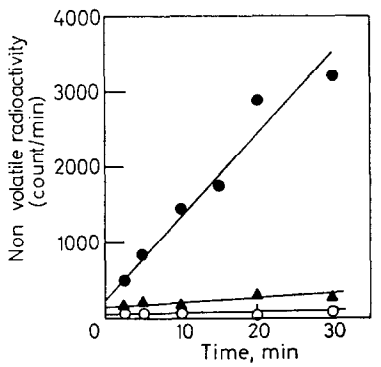

FIG. 2. Production of non-volatile radioactive compounds. Reaction mixture contained GSH (2mM); EDTA (4mM); [U-^{14}C] EDB (3mM) in phosphate buffer (pH 7·4), and enzyme preparation 0·5 ml which is equivalent to 140 mg liver; in a total volume of 6·5 ml. Incubation, 30 min. ●—enzyme + GSH + EDB; ▲—boiled enzyme + EDB; ○—GSH + EDB.

Enzyme activity was determined simultaneously in liver and kidney homogenate to compare the activities in the two tissues. The mean reaction rate of five experiments was 1·7 ± 0·02 and 1·4 ± 0·05 μmole GSH consumed per 1 min per 1 g liver and kidney, respectively.

When L-cysteine was used as a substrate, it was found to react with EDB non-enzymically. These results agree with those reported by Johnson[7] on glutathione S-alkyl transferase.

In another series of experiments, [U^{14}C] EDB was used as a substrate and the non-volatile radioactivity was measured (method c).

Figure 2 represents the amount of the radioactive product formed during the reaction. A straight line was obtained by plotting counts against time. The blank containing EDB and enzyme had a certain radioactivity which rose slightly with the passage of time. Only traces of radioactivity were found in the blank containing GSH and EDB.

These results confirm the existence and the reaction rate of enzyme which was previously determined, by the Br production and thiol consumption methods.

Oxidized glutathione was determined in the reaction mixture, in the presence and absence of EDB. The results obtained are presented in Table 1.

TABLE 1. GSH DISAPPEARANCE AND GSSG FORMATION DURING ENZYMIC REACTION OF EDB AND GSH

Time (min)	GSH consumed (μg/ml)	GSSG formed in the presence of EDB (μg/ml)	GSSG formed in the absence of EDB (μg/ml)
10	140	6·8	10
20	255	18	22
30	320	27	34
45	400	34·4	40

Reaction mixture: GSH 2mM; EDTA 2mM; EDB 4mM; enzyme preparation 0·5 ml (10 mg protein); phosphate buffer, 0·1 M; pH, 7·4; total vol., 6·5 ml; temp., 37°.

In spite of the presence of EDTA, a slight oxidation of GSH was observed, which decreased in the presence of EDB. This may be attributed to its competitive reaction with thiol groups.

These results are an additional proof that the main loss of glutathione in this reaction is due to conjugation with EDB.

Identification of the reaction product in vitro

The compounds formed as a result of the enzyme-catalysed reaction between EDB and GSH were prepared using labeled EDB (see Methods) and examined by paper and thin layer chromatography in solvents A, B, C, D and E, and the identification was carried out by comparing the R_f's with synthetic reference samples and by co-chromatography. The samples eluted from chromatograms were oxidized with

TABLE 2. R_f VALUES OF EDB METABOLITES *in vitro* AND *in vivo*

Solvent	A	B	C	D	E	H_2O_2 treated A	B	C
Reference compounds								
Glutamic acid	0·32	0·33	0·31			0·30	0·33	0·31
Glycine	0·29	0·42	0·36			0·23	0·42	0·36
GSH	0·34	0·40	0·29					
GSSG	0·17	0·08	0·20	0·14	0·27			
$GSCH_2CH_2OH$	0·31	0·46	0·40	0·26	0·36	0·20	0·27	0·30
$GSCH_2CH_2SG$	0·19	0·61	0·20	0·10	0·09	0·10	0·12	0·16
S-(β-hydroxyethyl) mercapturic acid	0·68	0·83	0·71			0·33	0·55	0·60
S-(β-hydroxyethyl) cysteine	0·33	0·68	0·55			0·21	0·53	0·22
Extract of enzymic reaction *in vitro*								
$GSCH_2CH_2OH$	0·31	0·46	0·40	0·26	0·36	0·20	0·27	0·30
$GSCH_2CH_2SG$	0·19	0·61	0·20	0·10	0·09	0·10	0·12	0·16
EDB treated rat liver extract								
$GSCH_2CH_2SG$	0·19	0·61	0·20	0·10	0·09	0·10	0·12	0·16
$GSCH_2CH_2OH$ sulphoxide	0·20	0·27	0·30					
$GSCH_2CH_2OH$	0·31	0·46	0·40	0·26	0·36	0·20	0·27	0·30
Unidentified substance	0·32		0·37					
Kidney extract								
$GSCH_2CH_2OH$	0·31	0·46	0·40			0·20	0·27	0·30
S-(β-hydroxyethyl) mercapturic acid	0·68	0·83	0·71			0·33		0·60

Samples prepared as described in the text. Reference substance added to the sample and chromatographed. Hydrogen peroxide oxidation product also examined in one- and two-dimensional chromatography in solvents A, B, C. The spots related to the prepared compounds were radioactive and reacted positively with platinic acid. All spots except mercapturic acid were ninhydrin-positive.

hydrogen peroxide and the oxidation products were compared with the synthetic references after oxidation (Table 2).

S-(β-hydroxyethyl) glutathione was identified as the main metabolite. A second metabolite, present in small amounts, was identified as ethylene S-S′ bis glutathione. Solvents B and C caused partial oxidation of these compounds. The compounds were radioactive and gave positive reactions with platinic acid and ninhydrin.

The radioactivity of $GSCH_2CH_2OH$ eluted from chromatogram was found to contain 93 per cent of the total radioactivity; the remaining 7 per cent was found in $GSCH_2CH_2SG$. The latter product may be formed in a non-enzymic process.

Identification of metabolites in vivo

The results of radiochromatogram scanning of liver and kidney samples from alcohol extracts, are shown in Fig. 3. In the liver samples, two peaks were found with R_f's of 0·19 and 0·31. In the corresponding kidney samples, two peaks were found with R_f's of 0·31 and 0·68.

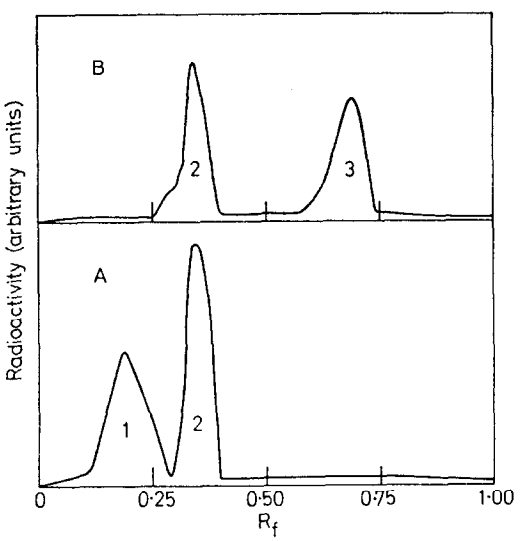

FIG. 3. Radiochromatograms of aliquots of alcohol extracts of liver (A) and kidney (B), obtained from rat given [U-^{14}C] EDB. The chromatograms were run in solvent butan-1-ol–acetic acid–water (75:21:42 v/v). The radioactive compounds in each peak were identified as follows: 1, $GSCH_2CH_2SG$ and $GSCH_2CH_2OH$ sulphoxide; 2, $GSCH_2CH_2OH$; 3, S-(β-hydroxyethyl) mercapturic acid.

When examined by two-dimensional chromatography in solvents A and C, peak No. 1 was found to contain $GSCH_2CH_2SG$ and oxidized $GSCH_2CH_2OH$. By using the same two solvents, peak No. 2 was found to contain mainly $GSCH_2CH_2OH$ and traces of an unidentified substance; and peak No. 3 contained S-(β-hydroxyethyl) mercapturic acid.

The total radioactivity recovered in the liver and kidney of an EDB-treated rat was 83,560 counts/min. 52,770 counts/min corresponding to 63 per cent of the total, were found in the liver. This amount represents 6 per cent of the radioactive material given to the rat.

DISCUSSION

The molar equivalent amounts of glutathione consumed and two Br ions formed showed a stoichiometric reaction of glutathione and EDB *in vitro*. These results prove that the two bromine atoms cannot be displaced simultaneously by the glutathione molecule in the enzymic reaction. One EDB molecule may react with one glutathione molecule to form an unstable intermediate complex product, which reacts with water to form the S-(β-hydroxyethyl) glutathione as shown in the following scheme.

$$GSH + BrCH_2CH_2Br \longrightarrow \overset{+}{G}SCH_2\overset{\longrightarrow}{C}H_2S\ \overset{-}{Br} + HBr$$

$$\Big\downarrow HOH$$

$$GSCH_2CH_2OH + HBr$$

The relatively higher concentration of water than glutathione in the reaction medium may be responsible for hydrolysis, and determines the end product of the reaction. The formation of $GSCH_2CH_2SG$ seems to result from a slow non-enzymic reaction.

The traces of GSSG found during the reaction prove that no enzymic- or auto-oxidation of glutathione occurs in the incubation media.

The enzyme responsible for the coupling of alkylhalide to glutathione was described in detail by Johnson,[7] and named glutathione S-alkyltransferase; glutathione S-aryltransferase was found by Booth *et al.*[6]

The identification of S-(β-hydroxyethyl) glutathione, S-(β-hydroxyethyl) glutathione sulphoxide and S-S' ethylene bis glutathione in the tissue, proves that glutathione is the main source of thiol groups for mercapturic acid formation, in the detoxification reactions. This phenomenon was also observed by other authors.[3,7-9,19]

The sulphoxide compounds found in the liver of EDB-treated animals are probably formed during the metabolic process. Sulphoxides of cysteine derivatives were isolated and identified by Barnsley *et al.*[18] and Sklan and Barnsley.[20]

The enzymic reaction between EDB and glutathione takes place primarily in the liver, and to a lesser extent in the kidney, resulting in the formation of S-(β-hydroxyethyl) glutathione and its sulphoxide. The later degradation of these compounds to the final products: S-(β-hydroxyethyl) mercapturic acid[2] and S-(β-hydroxyethyl) mercapturic acid sulphoxide,[21] occurs mainly in the kidney[22-24] and involves more than one enzyme.

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