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THE ROLE OF GLUTATHIONE CONJUGATION IN THE MUTAGENICITY OF 1,2-DIBROMOETHANE

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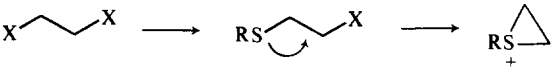
Abstract—Two mechanisms for the toxic actions of 1,2-dibromoethane have been postulated, both of which involve biotransformation. The first is oxidation to 2-bromoacetaldehyde, a highly reactive substance, the second a possible direct conjugation to glutathione, giving rise to a reactive half-mustard. It was the purpose of this investigation to determine to what extent these two reactive species are responsible for the mutagenicity of 1,2-dibromoethane. To assess quantitatively the importance of the conjugation to glutathione *in vivo*, rats were administered single doses of 1,2-dibromoethane; 30–55 per cent of the dose was excreted as mercapturic acid. The conjugation of 1,2-dibromoethane to glutathione was also studied *in vitro*. Specific activities of the metabolizing systems used in the mutagenicity experiments were determined. The mutagenicity of 1,2-dibromoethane towards *Salmonella typhimurium* TA100 was considerably enhanced by the addition of 100,000 *g* supernatant fraction, whereas the addition of microsomes had no effect, indicating that the primary glutathione adduct is responsible for the mutagenic effect. As a model for the mutagenic intermediate, *S*-2-bromoethyl-*N*-acetyl-cysteine methyl ester was synthesized. This proved to be a very reactive and highly mutagenic compound, which can be further metabolized and thereby detoxified by glutathione conjugation. A similar phenomenon is likely to occur in the mutagenicity test with 1,2-dibromoethane, where after an initial rise in the number of mutants with increasing amounts of glutathione, the number of mutations decreases again. These results clearly indicate that glutathione conjugation plays an important role in the mutagenicity of 1,2-dibromoethane.

1,2-Dibromoethane (DBE)† is widely used as an insecticide, nematocide, fungicide and gasoline additive [1]. A number of adverse effects of this compound have been reported, notably its mutagenicity towards bacteria [2] and carcinogenicity towards rats and mice [3]. Although its biotransformation pathways have been studied quite extensively, quantitative data are scarce [4–7]. Glutathione *S*-transferases seem to play an important role, giving rise to mercapturic acids [4] and, *in vitro*, to glutathione adducts [7], double glutathione adducts [7] and ethylene [8].

Opinions differ as to the identity of the species giving rise to mutagenic and carcinogenic effects. There are indications that the ultimate reacting species is 2-bromoacetaldehyde, supposedly formed via the mixed function oxidase system [9–10]. Recently, however, evidence was obtained that the GSH-*S*-transferases might be involved in the formation of the reactive intermediates from 1,2-dihalogen compounds. This has been shown for 1,2-dichloroethane [11] and *cis*-1,2-dichlorocyclohexane [12]. Particularly in the latter case, evidence was presented for the formation of mustard-type intermediates in which the sulfur atom of the initially formed conjugate reacts intramolecularly to give a thiiranium ion by eliminating the halogen from the neighbouring carbon atom.



It was the purpose of this investigation to ascertain the role of conjugation to GSH in the mutagenicity of DBE, compared to a possible activation via oxidation. Firstly, the importance of the mercapturic acid pathway in the metabolism of DBE was studied quantitatively *in vivo* in rats. Secondly, the conjugation of DBE to GSH was studied *in vitro* to define the GSH-*S*-transferase activity of the enzyme preparations used in the mutagenicity experiments that were performed. Thirdly, mutagenicity of DBE was tested towards *Salmonella typhimurium* TA100 in the presence of different rat enzyme preparations. Finally, the properties of a model compound for the possible reactive intermediate of DBE, *S*-2-bromoethyl-glutathione, the methylester of *S*-2-bromoethyl-*N*-acetyl-cysteine was studied.

MATERIALS AND METHODS

1,2-Dibromoethane. This was obtained from Baker Chemicals. It was purified by preparative gas–liquid chromatography (g.l.c.) and checked before use to be 99.9+ per cent pure by g.l.c.

† Abbreviations used: GSH, glutathione; DBE, 1,2-dibromoethane; DMSO, dimethylsulfoxide.

S-2-Hydroxyethyl-N-acetyl-cysteine (1). To a solution of 1.12 g (0.02 mole) KOH in 50 ml of methanol was added 1.63 g (0.01 mole) *N*-acetyl-L-cysteine. After 10 min stirring, a solution of 1.24 g (0.01 mole) bromoethanol in 25 ml of methanol and this mixture was refluxed for 4 hr in an argon atmosphere. After cooling, the solution was neutralized with a dry solution of HCl in methanol. The solvent was evaporated *in vacuo* and 50 ml of acetone was added to the residual slurry. The salts were filtered off and the solvent was evaporated *in vacuo*. The residual oil was dried over P_2O_5 until no H_2O peak was visible in the NMR spectrum, yielding 82 per cent (1.7 g) of colorless oil. NMR: ($CDCl_3/CD_3OD$; TMS), 2.02 (s, 3H), 2.68 (t, 2h, J = 7 Hz), 2.94 (m, 2H), 3.68 (t, 2H, J = 7 Hz), 4.56 (m, 1H).

S-2-*Hydroxyethyl*-N-*acetyl-cysteine methylester* (2). The reaction was performed as described for (1). Instead of neutralizing the reaction mixture, it was acidified with HCl in methanol and stirred for 15 min. Then the solution was brought to pH 4–5 with a solution of sodium methanolate in methanol, the salts filtered off and the solvent evaporated *in vacuo* to give 2 g (90 per cent) of a pale yellow oil. NMR: ($CDCl_3$, TMS), 2.04 (s, 3H), 2.78 (t, 2H, J = 7 Hz), 2.90 (m, 1H, disapp. on add. of D_2O), 3.04 (d, 2H, J = 6 Hz), 3.82 (s, 3H), 3.84 (t, 2H, J = 7 Hz), 4.80 (m, 1H), 6.86 (m, 1H). ms: 221 (m, ±), 203 (45%), 189 (5%), 171 (10%), 162 (20%), 144 (100%).

S-2-*Bromoethyl*-N-*acetyl-cysteine methylester* (3). Compound (2) [1g (4.5 mmoles)] was dissolved in a concentrated solution of HBr in methanol and stirred for 30 min. The solvent was evaporated *in vacuo* and the residue purified over a silica gel column with ethylacetate as eluent, yielding 0.6 g (47 per cent) of a white crystalline solid, m.p. 47–49° NMR: ($CDCl_3$, TMS), 2.04 (s, 3H), 2.90 (t, 2H, J =7 Hz), 3.00 (d, 2H, J=6 Hz), 3.44 (t, 2H, J=7 Hz), 3.76 (s, 3H), 4.80 (m, 1H), 6.70 (m, 1H). ms= 283–285 (m, ±), 282–284 (0.5%), 224–226 (10%), 204 (85%), 182–184 (20%), 176 (30%), 167 (25%) 145 (100%).

S-*Benzyl*-N-*acetyl-cysteine* (4). The reaction was performed as described for (1) yielding 85–95 per cent of a white crystalline solid, m.p. 138–139° NMR: ($CDCl_3/CD_3OD$), 1.98 (s, 3H), 2.88 (d, 2H, J=6Hz), 3.70 (s, 2H), 4.64 (t, 1H, J = 6Hz), 7.22 (s, 5H). ms= 253 (m^+, 5%), 194 (17%), 171 (10%), 91 (100%).

Reaction of (3) *with* p-*nitrothiophenol in water*. Compound (3) [0.07 g (2.4 mmoles] dissolved in 5 ml of dry tetrahydrofuran was added to a solution of 0.4 g (2.6 mmoles) *p*-nitrothiophenol in 15 ml of water (pH 6). The orange solution became pale yellow in 15 min and was extracted twice with 15 ml of chloroform. The chloroform layer was dried on $MgSO_4$ and the solvent evaporated *in vacuo*. The residue was chromatographed on a silica gel column with ethylacetate/methanol (10:1) as eluant. The first fraction yielded 0.4 g of the *p*-nitrothiophenol adduct (5), (45 per cent), the second fraction 0.3 g of (2) formed by reaction of (3) with water. NMR (5): ($CDCL_3$, TMS), 2.06 (s, 3H), 2.82 (m, 2H), 3.02 (m, 2H), 3.18 (m, 2H), 3.76 (s, 3H), 4.80 (m, 1H), 6.78 (m, 1H), 7.34 (d, 2H, J=9 Hz), 8.14 (d, 2H, J = 9 Hz).

Animal experiments. One millilitre of a solution of DBE (for doses see Fig. 2, range: 9.4–329 mg/kg) in arachis oil was given orally by stomach tube to male rats of the laboratory-bred SPF Wistar strain, weighing about 200 g. The rats were kept in stainless steel metabolism cages and urine was collected for 24 hr. The animals were fasted during the experiments, but not before, and they had free access to water.

Determination of S-2-*hydroxyethyl*-N-*acetyl-cysteine in urine*. Urine was freeze-dried, taken up in 10 ml methanol–ether (2:3) and 1 ml of this solution was treated with an ethereal solution of diazomethane, taken to dryness again and dissolved in 0.5 ml ethylacetate. Before freeze-drying, 1 mg of the internal standard *S*-benzyl-*N*-acetyl-cysteine was added as a solution in 100 µl water. Ethylacetate solution (0.1 µl) was injected into a Carlo Erba 2300 gas chromatograph, equipped with an 8 m capillary column, wall-coated with 5% Carbowax 20 M, and a flame ionization detector (conditions: injection port 225°, detector 280°, column 175°, carrier gas: hydrogen). A definite identification of the mercapturic acid was performed using an LKB-2091-2130 (EI/CI) gas–liquid chromatograph–mass spectrometer–com-

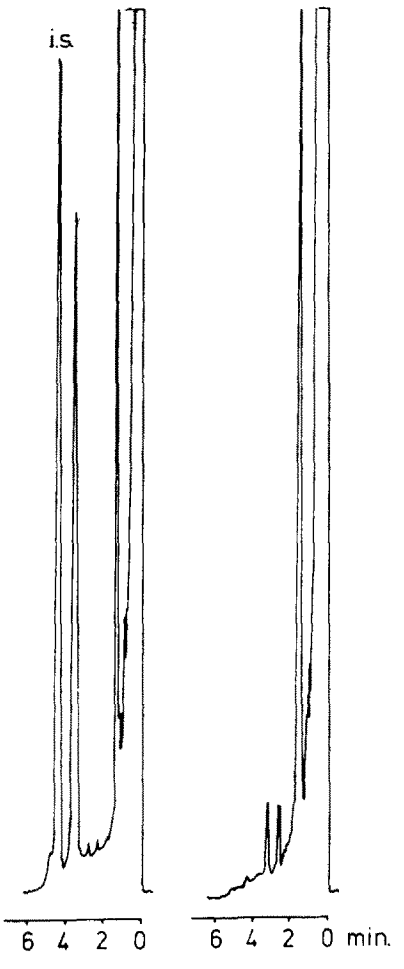


Fig. 1. A representative g.l.c. trace of *S*-2-hydroxyethyl-*N*-acetyl-cysteine methylester from urine. On the right is a g.l.c. trace of blank urine extract.

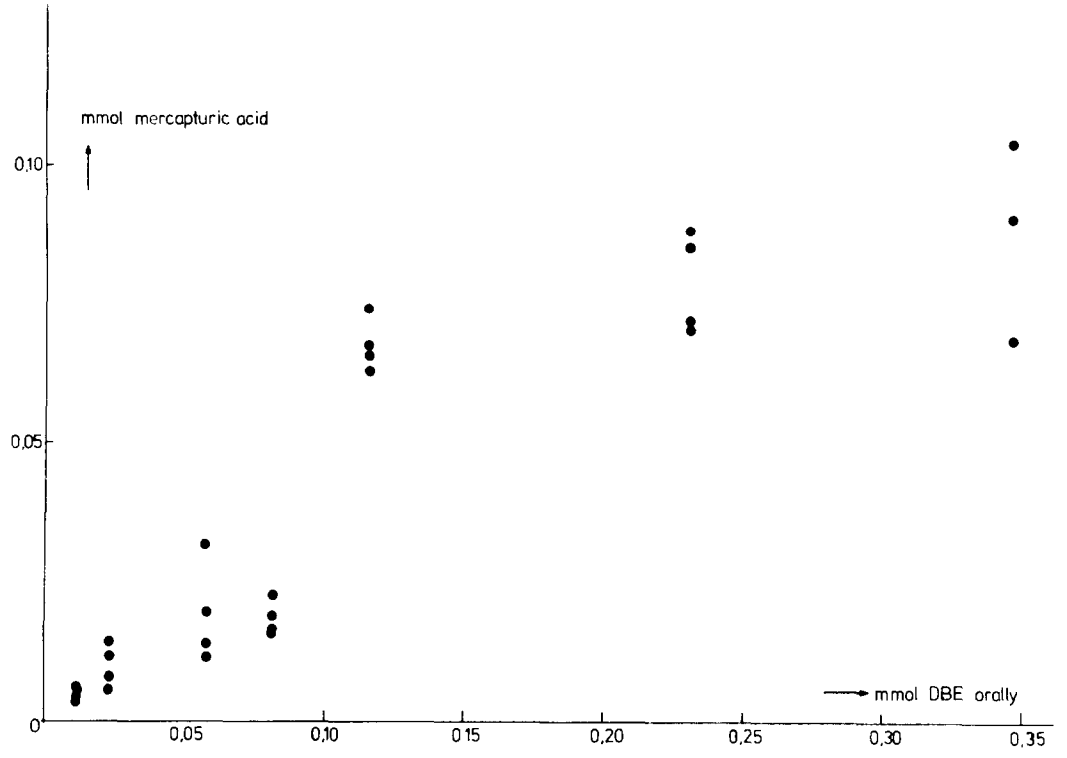


Fig. 2. Excretion of mercapturic acid as a function of dose. Each point represents the results obtained from one rat.

puter combination, equipped with the same type of column.

Enzyme preparations. Male rats of the laboratory-bred SPF Wistar strain weighing about 200 g were induced by pretreating them intraperitoneally with a solution of Aroclor 1254 in 0.5 ml sesame oil (75 mg/kg), 5 days before they were killed. They were starved overnight and killed by cervical dislocation. The liver was perfused *in situ* via the portal vein with 0.15 M KCl, removed and washed. To 100 g liver 220 ml 0.15 M KCl was added and homogenization was performed with a Potter homogenizer. The homogenate was centrifuged for 20 min at 9000 *g*. Part of the supernatant fraction thus obtained was frozen directly at −80° and kept at that temperature until use. Another part was centrifuged for 1 hr at 100,000 *g*. The 100,000 *g* supernatant fraction (cytosol) thus obtained was filtered over glass wool, frozen at −80° and kept at that temperature. The microsomal pellet was washed with 0.15 M KCl, recentrifuged and suspended in 50 ml 0.15 M KCl. This suspension was frozen at −80° and kept at that temperature. Cytochrome P-450 content was determined from the CO binding spectrum [13]. Protein content of all fractions was determined according to Lowry *et al.* [14], using bovine serum albumin as a standard. All handlings were carried out at 0–5°.

Determination of GSH-S-transferase activity. GSH-*S*-transferase activity towards DBE was determined according to the procedure of Baars *et al.* [15] with slight modifications. The standard assay consisted of 1.0 ml 0.04 M Tris–HCl, 3 mM EDTA, pH 8.0; 0.25 ml 100,000 *g* supernatant fraction containing 9 mg protein per ml; GSH 0.10 ml in distilled water (final concentration 0.76 mM); DBE in 0.050 ml absolute ethanol (final concentration 4.1 mM), giving a total incubation volume of 1.5 ml. Mixtures were preincubated for 2 min at 37° and the reaction was started by adding the substrate, DBE. Incubation took place at 37° in a shaking waterbath for 15 min. Under these conditions product formation was linear with time and protein concentration. The reaction was stopped by adding 0.10 ml 33% aqueous trichloroacetic acid followed by centrifugation at 600 *g* for 5 min. Appropriate controls in each experiment served as blanks and for the determination of non-enzymic reactions. Conjugation was calculated by measuring the unreacted GSH with Ellman's reagent; 0.1 ml of the clear supernatant fluid was added to 1.5 ml reagent (0.5 mM 5,5'-dithio-bis-2-nitrobenzoic acid in 0.1 M phosphate buffer, pH 6.5). After 5 min standing the absorption at 412 nm was measured. Assuming Michaelis–Menten kinetics, apparent K_m and V_{max} values were calculated according to Baars *et al.* [15].

Mutagenicity experiments. The experiments were performed by a modification of the assay as described by Ames [16]. The indicator bacteria (*Salmonella typhimurium* TA100) were grown overnight (16 hr) in nutrient broth, on a rotary shaker, and had reached the stationary phase at the end of the incubation period. The enzyme preparations were the same as described above. The reaction mixtures consisted of 0.85 ml 0.06 M phosphate buffer (pH 7.4,

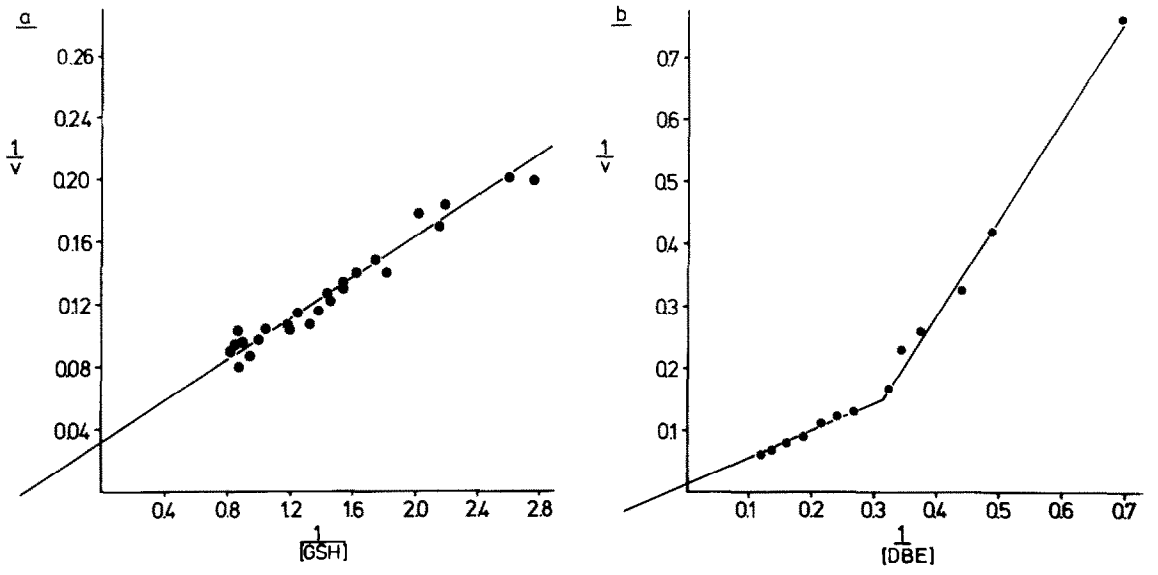


Fig 3. Lineweaver–Burk plots showing the effect of substrate concentration on the reaction rate of rat liver GSH-*S*-transferase activity. Panel a: Variation of GSH at a concentration of 4.1 mM DBE. Panel b: Variation of DBE at a concentration of 0.69 mM GSH.

0.4% saline), 0.25 ml overnight culture of TA100, 0.20 ml of a cofactor solution, pH 7.4 [17] (for microsomes and 9000 *g* supernatant fractions), 0.20 ml of a solution of GSH in 0.06 M phosphate buffer, pH 7.0, final concentration 0.76 mM (for 100,000 and 9000 *g*), 0.15 ml of metabolizing system and the substance DBE in 0.050 ml ethanol. This mixture was incubated for 10 min at 37° in a rotary shaker. Then 10 ml soft agar (48°) was added, thoroughly mixed and 3 × 3 ml poured over his$^+$ mutation plates. The plates were incubated for 48 hr and the number of his$^+$ revertants was determined.

RESULTS

In vivo *experiments*

Gas chromatography proved a satisfactory method to quantify the main GSH-derived metabolite of DBE: *S*-2-hydroxyethyl-*N*-acetyl-cysteine. In Fig. 1 a representative gas chromatogram is shown. There are no interfering peaks and satisfactory calibration curves were established. In this way the cumulative excretion of the mercapturic acid was determined. All of the metabolite was excreted within 24 hr. As is shown in Fig. 2, up to a dose of 0.15 mmole DBE per rat, there is roughly a proportionate increase of mercapturic acid excretion with dose, whereas at higher dose levels this amount seems to reach a plateau. In the lower dose range 30–55 per cent of the dose is excreted as mercapturic acid.

In vitro *experiments*

GSH-S-transferase activity. The activity of the GSH-*S*-transferases towards DBE was determined according to Baars *et al.* [15], using Ellman's reagent to measure the residual GSH. In preliminary experiments the optimal conditions had been established for this determination in rat liver cytosol and 9000 *g* supernatant fraction. The preparations used in the mutagenicity experiments had the following activities: 9000 *g* supernatant fraction 7.2 nmoles/min/mg protein and 100,000 *g* supernatant fraction (cytosol) 10.9 nmoles/min/mg protein. Under the conditions used, spontaneous conjugation accounted for 16 per cent of the total conjugation. The kinetic behaviour of the enzymatic reaction is illustrated in Fig. 3. By varying the GSH concentration a $K_m$app. (GSH) of 1.5 ± 0.4 mM and V_{max}app. (GSH) of 27 ± 5 nmoles/min/mg protein could be determined, at a concentration of 4.1 mM DBE. It was not possible to work at enzyme saturation levels of DBE, owing to its limited solubility in water. Variation of the DBE concentration (at a concentration of 0.69 mM GSH) gave, instead of a hyperbolic, a sigmoid curve, so that in the Lineweaver–Burk plot two straight lines were observed (Fig. 3b). From the lower line a $K_m$app. (DBE) of 73 ± 20 mM and V_{max} app. (DBE) of 160 ± 30 nmoles/min/kg protein could be determined.

Interaction of S-2-bromoethyl-N-acetyl-cysteine methylester with sulfur nucleophiles. As a model for the initially formed glutathione conjugate from DBE, *S*-2-bromoethyl-*N*-acetyl-cysteine methylester was synthesized and its reactivity studied. When added to a solution of *p*-nitrothiophenolate anion in water (pH 6), 45 per cent reacted with the sulfur nucleophile and 55 per cent with water. The products of these reactions were isolated and characterized (see Materials and Methods). The model intermediate also reacts with GSH: 15 min after addition of 10 μmoles *S*-2-bromoethyl-*N*-acetyl-cysteine methylester in 0.5 ml DMSO to 10 ml of a 0.76 mM solution of GSH in water (pH 7.4) 60 per cent of the GSH had disappeared (as determined with Ellman's reagent).

Mutagenicity experiments

Metabolic activation of DBE. In Fig. 4 it is shown that DBE is somewhat mutagenic towards *Salmonella* TA100 without mammalian metabolic activa-

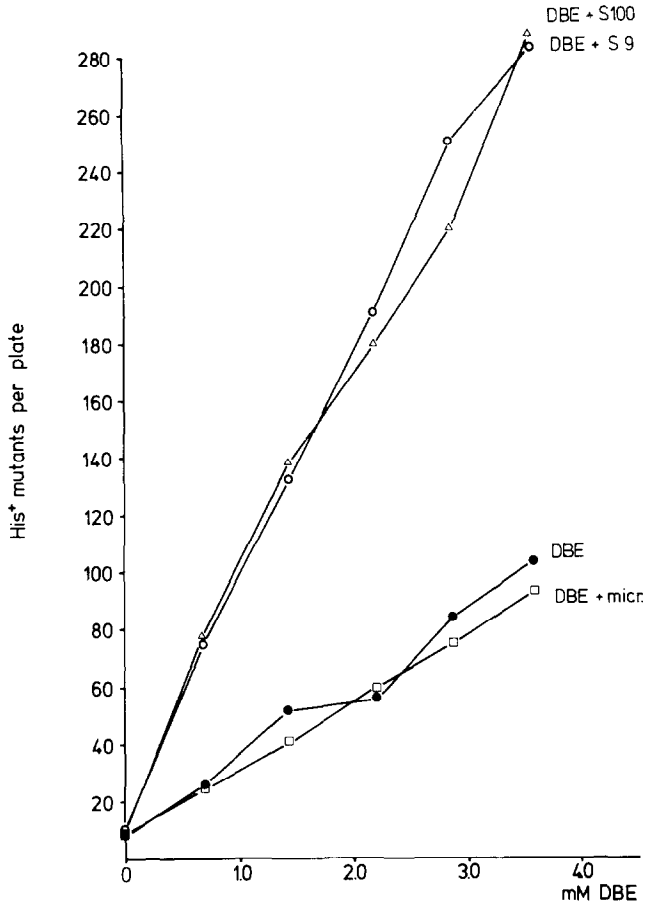


Fig. 4. Mutagenic activity of DBE under different conditions, The amount of protein added was for 9000 *g* 40.3 mg/ml (sp. act. GSH-*S*-transferases 7.2 nmoles/min/mg protein); 100,000 *g*: 17.9 mg/ml (sp. act. GSH-*S*-transferases 10.9 nmoles/min/mg protein); microsomes: 10.6 mg/ml (containing 1.6 nmoles cytochrome-P 450/mg protein).

tion. However, when rat liver 9000 *g* supernatant fraction is added, there is a considerable increase in the number of his$^+$ revertants, indicating a metabolic enhancement of DBE mutagenicity. To check in which cellular fraction this activation is located, the 9000 *g* supernatant was fractionated into a 100,000 *g* supernatant fraction and a microsomal fraction. When these were used separately, the microsomes did not enhance the number of revertants above that caused by DBE itself. On the other hand, the 100,000 *g* supernatant fraction, containing the GSH-*S*-transferase activity, potentiates the mutagenicity quite extensively.

Mutagenicity of S-2-*bromoethyl*-N-*acetyl-cysteine methylester*. In Fig. 5 the results of a typical experiment with the synthetic model for the first glutathione adduct of DBE, *S*-2-bromoethyl-*N*-acetyl-cysteine methylester, are presented. For comparison, DBE was tested under the same conditions. These data clearly show that, upon formation of a GSH-conjugate possessing a mustard-like moiety, a drastic increase in the number of mutations is indeed observed.

Influence of GSH on the mutagenicity of S-2-*bromoethyl*-N-*acetyl-cysteine methylester*. In Fig. 5 the influence of GSH on the mutagenicity of the model intermediate is shown. In accordance with the results obtained in the reactivity tests, addition of GSH lowers the mutagenicity, obviously because GSH scavenges the intermediate thiiranium ion. In Fig. 6 the influence of GSH concentration on the mutagenicity of DBE is shown. As expected, the addition of GSH initially produces an increase in the number of his$^+$ revertants. However, at GSH concentrations higher than ± 0.8 mM, the mutagenicity is lowered. This is in agreement with the results obtained with the synthetic intermediate: the extra addition of GSH scavenges the reactive intermediates formed through the first conjugation. The same effect was observed without added activating system (Fig. 6).

DISCUSSION

The conjugation to GSH plays an important role in the metabolism of DBE. The most abundant final metabolite of this pathway is the mercapturic acid, *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine, which comprises up to 55 per cent of the administered dose.

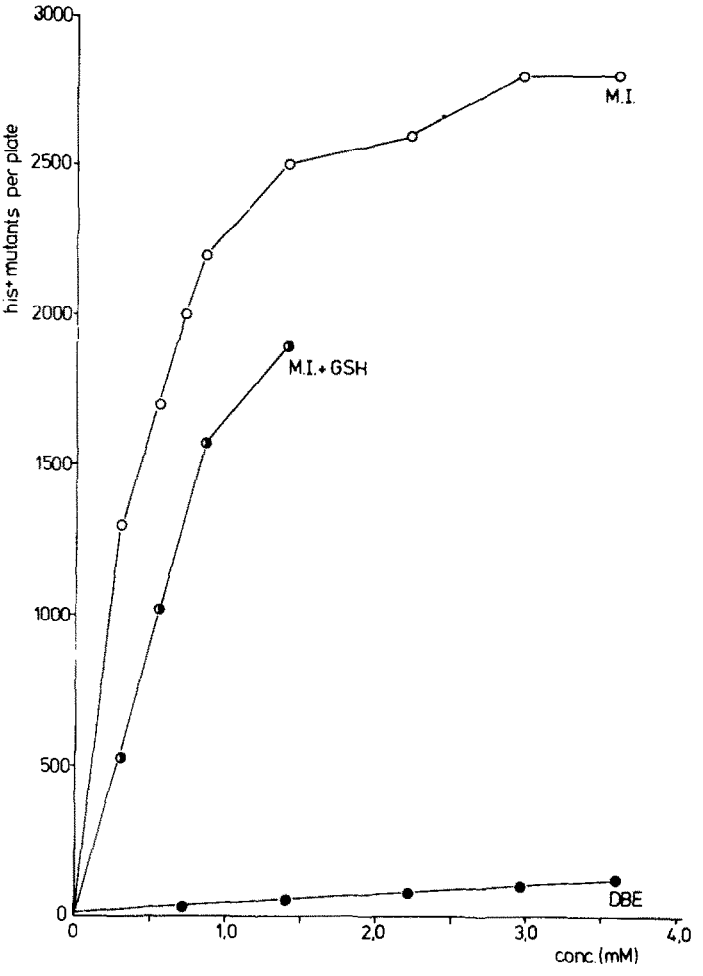


Fig. 5. Mutagenic activity of *S*-2-bromoethyl-*N*-acetyl-cysteine methylester (M.I.) without (○) and with (●) glutathione (0.76 mM), as compared to DBE itself (●).

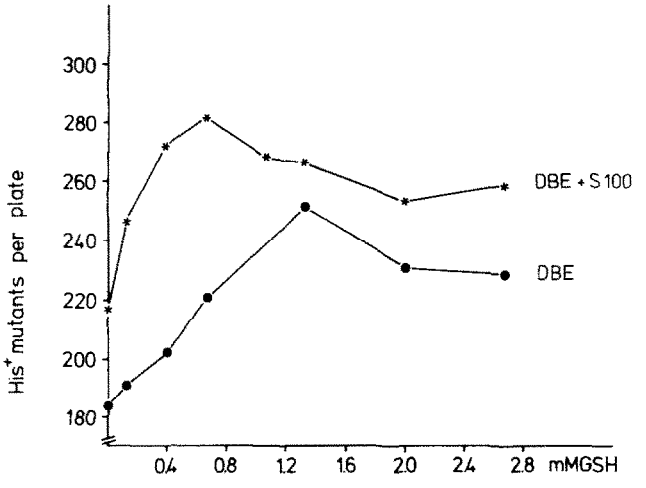


Fig. 6. Influence of the concentration of GSH on the mutagenic activity of DBE with and without metabolic activation. Concentration of DBE 3.6 mM in both cases; 100,000 *g* contains 15 mg protein/ml (specific activity 10.9 nmoles/min/mg protein).

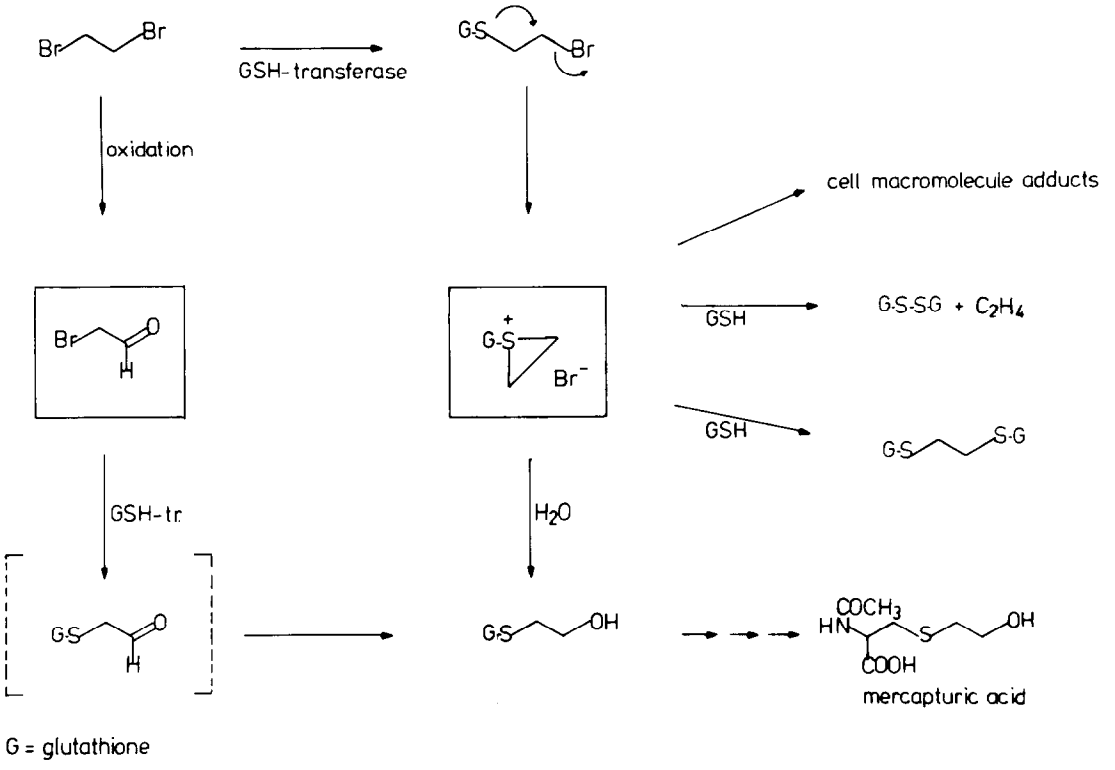


Fig. 7. The possible metabolic pathways of DBE. Both give rise to the same mercapturic acid, but via different reactive intermediates.

Since a number of related metabolites have been identified *in vivo* — the non-acetylated cysteine adduct [4], *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine-S-oxide [5], *S*-carboxymethyl-L-cysteine and thiodiglycolic acid [18] — the total amount of DBE conjugated to GSH will be even higher.

It is of interest that the amount of mercapturic acid excreted reaches a maximum at relatively high dose levels (Fig. 3). Most likely this is due to exhaustion of the GSH supply, which is around 0.05–0.1 mmole per total rat liver [19, 20]. Since the amount of mercapturic acid formed at dose levels above 0.15 mmole DBE is 0.06–0.1 mmole per rat, presumably the amount of GSH available is exhausted and *de novo* synthesis cannot keep up with the amount needed. Differences between rats could then reflect differences in available GSH and GSH precursors.

Nachtomi [7] was the first to show *in vitro* the enzymic nature of the conjugation of GSH to DBE, which was confirmed by Hill *et al.* [9], who measured apparent K_m and V_{max} values for DBE. Livesey and Anders [8] recently showed that the enzymic reaction of GSH with DBE has a more complex character. A second reaction of GSH with the first-formed conjugate can lead to formation of ethylene and GSH-disulfide or, as Nachtomi [7] reported, a double conjugate. The explanation of the sigmoid curve obtained under our conditions in the Michaelis–Menten plot for the variation of DBE probably is that we are dealing here with a very complex mixture of reactions. More detailed studies, in which all reaction products are measured and perhaps purified enzyme systems are used, are needed to clarify this phenomenon. Investigation of the chemical reactivity of the synthetic model for the first formed conjugate, *S*-2-bromoethyl-*N*-acetyl-L-cysteine methylester, revealed that it is a strong alkylating agent. It reacts via an intermediate thiiranium ion with the model nucleophile *p*-nitrothiophenol and water, and also with GSH under the conditions used in the GSH-*S*-transferase assay. No formation of mixed disulfide was observed in the reaction with *p*-nitrothiophenol, which would have pointed to the formation of ethylene [8], but this could have been a minor side product. Because of its reactivity we expected to find that the model conjugate was a very potent mutagen, as was found previously with its cyclic analogs [12].

The oxidative pathway in the metabolism of DBE gives bromoacetaldehyde as its first product, as shown by Hill *et al.* [9] and Banerjee *et al.* [10]. This compound is very similar to the known mutagen chloroacetaldehyde [21], although it was reported as non-mutagenic by Rosenkranz [22]. Covalent binding to macromolecules was found as the result of oxidative metabolism of DBE [9–10]. Conjugation to GSH appears to be a real detoxification reaction in this case.

The conclusion from the foregoing is that there are two likely pathways for the formation of *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine, both of which give rise to a reactive intermediate. These routes are depicted in Fig. 7. (1) Direct conjugation to GSH, giving rise to reactive half-mustards. Subsequent reaction with

water via the intermediate thiiranium ion then leads to the 2-hydroxyethyl adduct. Reaction of the thiiranium ion with a second molecule of GSH would lead to either a double conjugate [7] or ethylene [8], depending on the mode of attack of the GSH molecule. (2) Oxidation to 2-bromoacetaldehyde, followed by conjugation to GSH. Reduction of the aldehyde grouping leads to the 2-hydroxyethyl adduct. The primary aldehyde adduct is also formed during the metabolism of vinylchloride [23] and, by analogy, *S*-carboxymethyl-cysteine and thiodiglycolic acid are also final products of this pathway.

DBE is a well-known mutagen [24], and its mutagenic activity can be enhanced by the addition of rat liver S9 fraction. From experiments of Rannug *et al.* on 1,2-dichloroethane [11] and of Van Bladeren *et al.* on *cis*-1,2-dichlorocyclohexane [12] it is known that activation through conjugation to GSH can be shown by adding S100, containing the cystosolic GSH-*S*-transferases, instead of the S9 fraction as the metabolizing system. For DBE only the S100 gives rise to enhanced mutagenicity, whereas the microsomal fraction appears to have no effect. From these results it is clear that the mutagenicity of DBE is caused by the formation of reactive GSH-conjugates. The fact the DBE is also mutagenic by itself could mean that spontaneous conjugation to sulfur and nitrogen nucleophiles occurs, giving rise to the same type of half-mustards. However, it cannot be excluded that the *Salmonella* bacteria possess a GSH-*S*-transferase system of their own. Although the total GSH-*S*-transferase activity in the added S9 fraction is higher than in the S100 (290 as compared to 195 nmoles/min/ml) the amount of mutations caused is not higher, presumably because the larger amount of protein added (40.3 mg/ml as compared to 17.9 mg/ml) scavenges the reactive intermediates.

Because the reactive intermediate GSH conjugate can react with a second molecule of GSH, addition of increasing amounts of GSH in the mutagenicity test has two effects. Initially the mutagenicity is enhanced, but at higher concentrations of GSH the reactive intermediates are scavenged and the mutagenicity is lowered (Fig. 6).

In conclusion, it is clear that, although oxidative metabolism of DBE gives rise to covalent binding *in vitro*, the mutagenicity of DBE towards *Salmonella typhimurium* TA100 is caused by conjugation to GSH. To what extent these two reactive intermediates are responsible for the adverse effects of DBE *in vivo* remains to be clarified.

The findings presented in this paper have implications for other compounds possessing vicinal halogen atoms: if no steric considerations hinder the formation of adduct or thiiranium ion, they can all be activated by the mechanism described in this paper.

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