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GLUTATHIONE S-TRANSFERASES CATALYZED CONJUGATION OF 1,4-DISUBSTITUTED BUTANES WITH GLUTATHIONE IN VITRO

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SUMMARY: Rat liver glutathione S-transferases catalyzed the conjugation of 1,4-diiodobutane with glutathione in vitro. The reaction followed saturation kinetics and was dependent on the concentration of the enzyme, substrate and glutathione in the incubation media. S-Benzylglutathione inhibited the enzymatic conversion of 1,4-diiodobutane to product. The cyclic sulfonium compound, γ-glutamyl-β-(S-tetrahydrothiophenium) alanyl-glycine was identified as the product of this conjugation reaction. This product was stable under physiological conditions in presence of rat liver cytosol but rapidly and quantitatively decomposed at pH $\geq$ 12 to give tetrahydrothiophene.

Introduction: Busulfan (1a) is a bifunctional alkylating agent which is used in the treatment of patients with chronic myelogenous leukemia. The major urinary metabolite obtained after administration of the drug to rats and rabbits was 3-hydroxytetrahydrothiophene-1,1-dioxide (5) (1). On the basis of in vitro studies, Roberts and Warwick proposed that in vivo busulfan reacted with cysteine or a cysteinyl moiety of a peptide to form a cyclic sulfonium compound which would decompose to tetrahydrothiophene (4). The tetrahydrothiophene would be oxidized first to tetrahydrothiophene-1,1-dioxide and then to 3-hydroxytetrahydrothiophene-1,1-dioxide. Attempts to form a cyclic sulfonium compound non-enzymatically from busulfan and cysteine were successful only under alkaline conditions. No attempt was made to determine if the cyclic compound was formed enzymatically. It appeared likely to us that the initial step in the metabolism of busulfan, and other 1,4-disubstituted butane, is conjugation with GSH catalyzed by glutathione S-transferases

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Abbreviations: CMR, carbon magnetic resonance; EDTA, ethylenediamine tetraacetic acid; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin layer chromatography.

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to give 3. Interestingly, a similar pathway has been proposed for the metabolism of vicinal dihalo compounds such as 1,2-dichloroethane and 1,2-dibromoethane. However, only indirect evidence exists to substantiate the formation of the ethylglutathione episulfonium intermediate (2,3). Therefore, we initiated a systematic investigation of the *in vitro* and *in vivo* metabolism of 1,4-disubstituted butanes. In this report we describe the results of studies conducted to delineate the role of the glutathione S-transferases in the metabolism of 1,4-disubstituted butanes *in vitro*. 1,4-Diiodobutane (1b) was used as the substrate in these studies.

MATERIALS AND METHODS: 1,4-Diiodobutane, tetrahydrothiophene, 5-bromopentane, 1,5-dibromopentane, and 1-bromo-4-chlorobutane were purchased from Aldrich Chemical Company. GSH and cysteine were obtained from Sigma Chemical Company. S-Benzylglutathione was previously synthesized in this laboratory (4). γ-glutamyl-β-(S-tetrahydrothiophenium) alanyl-glycine (3) was prepared by stirring 1-bromo-4-chlorobutane, GSH, and sodium hydroxide (3 equivalents) in H_2O/EtOH at room temperature for 45 minutes. The mixture was adjusted to pH 6.5 and allowed to stand at room temperature for 24 hours. Compound 3 was separated by using an AG 1-X2 (Bio-rad) ion exchange column and a gradient of 0.01-0.2 M ammonium acetate as the eluent. The structure of 5 was confirmed by high resolution NMR and CMR. The purity of the product was established by HPLC and TLC.

Enzyme Preparation: Rat liver cytosol (109,000 g supernatant) was prepared from a 20% homogenate in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA of livers obtained from male Sprague-Dawley rats (150-200 g). Rat liver glutathione S-transferases was purchased from Sigma. This preparation is a mixture of isozymes which is obtained by GSH-affinity chromatography according to the procedure of Simons and Vander Jagt (5). In some experiments the rat liver glutathione S-transferases preparation was passed through a 1.5 x 15 cm Bio-Gel P-10 column (Bio-Rad) in 0.1 M potassium phosphate buffer (pH 7.4) at 4°C to remove residual GSH (6).

The activity of the glutathione S-transferases was measured using 1-chloro-2,4-dinitrobenzene according to the procedure of Habig et al. (7). Protein was measured by a dye-binding assay (Bio-Rad Protein Assay Kit; Bio-Rad Laboratories) using bovine serum albumin as the standard (8). The mean specific activities of the rat liver cytosol was 0.073 μmol/min/mg protein and that of the rat liver glutathione S-transferases preparation was 11.0 μmol/min/mg.

Enzyme Incubations: Enzyme incubations were performed in a 0.1 M phosphate buffer (pH 7.4) containing 1 mM EDTA and the specified amount of GSH. The incubation solution (1 mL) was placed in a 13 x 100 mm screw-capped pyrex test-tube and the required amount of rat liver cytosol or glutathione S-transferases was added. In some experiments, S-benzylglutathione was added in 50 or 100 μM concentrations. The tubes were placed in a water bath at 37°C for 5 min. The reaction was started by the addition of a 25 μL of an alcoholic solution of 40 mM 1,4-diiodobutane (final ethanol concentration was 2.5%). Each tube was tightly capped with teflon-lined caps, vortexed for a few seconds and placed in a shaker bath at 37°C. After the specified time interval the reaction was quenched by immersing the test-tube in an ice water bath.

Determination of tetrahydrothiophene and 1,4-diiodobutane in incubation mixtures: A Perkin-Elmer 3920 B gas chromatograph equiped with a flame ionization detector and a Hewlett-Packard 3390A computing integrator were

used. A 3.6 m x 4.4 mm i.d. glass column packed with 20% Carbowax 20 M on 80/100 supelcoport at a column temperature of 130°C was used for the determination of tetrahydrothiophene and a 1.8 m x 2.2 mm i.d. glass column packed with 10% SP-2401 on 100/120 Supelcoport at a column temperature of 110°C was used for the determination of 1,4-diiodobutane (9). 5-Bromopentane and 1,5-dibromopentane were used as internal standards for the determination of tetrahydrothiophene and 1,4-diiodobutane, respectively. Correlation graphs were constructed using appropriate concentrations of tetrahydrothiophene or 1,4-diiodobutane.

A 0.5 mL of ice cold n-hexane, containing the internal standard, was quickly added to each of the tubes containing the quenched incubation mixtures and the tube was vigorously shaken for 10 seconds to extract any unreacted 1,4-diiodobutane. A 100 µl of 3 N sodium hydroxide was added, and after 5 minutes 0.5 mL of ice cold n-hexane, containing the internal standard was added. The mixture was shaken and the tubes were stored in the dark at 4°C until analysis. Aliquots of the n-hexane extracts were analyzed by GC.

Detection of 5 in incubation mixtures by TLC and HPLC.

The quenched incubation mixture was treated with 0.25 mL of 20% trichloroacetic acid at 4°C. A 0.5 mL of ice cold n-hexane was quickly added and the mixture shaken vigorously. The n-hexane layer was separated and the aqueous phase was centrifuged. The supernatant was divided into two aliquots and one of the aliquots was adjusted to pH $\geq$ 12 by the addition of 3 N sodium hydroxide solution. A 0.5 ml of n-hexane was added to each of the aliquots and shaken vigorously. The n-hexane phase was removed and analyzed by GC. The aqueous phase was centrifuged and the supernatent was adjusted to pH 7.4 and examined by HPLC and TLC.

TLC analysis was performed on Analtech RP-S plates using isopropyl alcohol:water:acetic acid (70:29:1) as the mobile phase. The dried plates were visualized with a ninhydrin spray.

Glutathione and its conjugates were analyzed by HPLC after pre-column derivatization with o-phthalaldehyde and 2-mercaptoethanol (10). The analysis was performed with a Rheodyne 7125 injector, a Waters Model M-6000 pump, a Shimadzu RF-530 fluorescence monitor, a Hewlett-Packard 3390 A integrator and a Regis Spherisorb S5-C_8 column. The o-phthalaldehyde derivatives were eluted with methanol/water (35:65) containing 0.01 M tetrabutylammonium bromide and 0.01 M sodium phosphate (pH 7.4).

RESULTS AND DISCUSSION: Incubation of 1,4-diiodobutane in a buffer containing GSH and rat liver cytosol resulted in the rapid disappearance of substrate. Adjusting the pH of the incubation mixtures, after the specified incubation periods, to pH $\geq$ 12 resulted in the formation of a volatile product which had the same retention time and mass spectrum as those of an authentic sample of tetrahydrothiophene on GC/MS analysis. Only a trace of tetrahydrothiophene was formed in the incubation mixtures prior to the addition of base suggesting that the product obtained from **1b** did not decompose to give tetrahydrothiophene under physiological conditions in presence of rat liver cytosol.

Replacing rat liver cytosol with partially purified rat liver glutathione S-transferases resulted in a similar disappearance of the substrate **1b** and the formation of tetrahydrothiophene after adjusting the incubation media to pH $\geq$

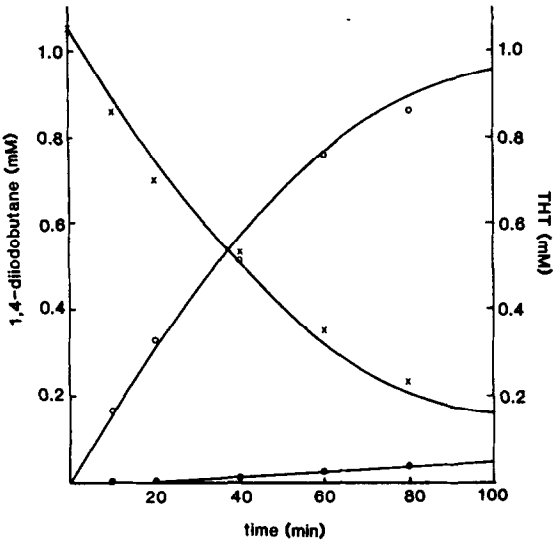


Figure 1. Time course of disappearance of 1,4-diiodobutane and appearance of Compound 3. 1,4-Diiodobutane (1.06 mM) was incubated in a 0.1 M phosphate buffer (pH 7.4) containing GSH (5 mM) and glutathione S-transferases (0.034 mg/mL) at 37°C. At various time intervals the tubes containing the incubation mixture were immersed in an ice-water bath and shaken with 0.5 ml n-hexane (containing the internal standard). The concentrations of 1,4-diiodobutane and tetrahydrothiophene (THT) in the n-hexane layer was determined by GLC. A 100 µL of 3N sodium hydroxide solution was added to each tube and after 5 minutes the mixture was reshaken. The concentration of tetrahydrothiophene (THT) in the n-hexane extracts was again determined by GLC. (x) Concentration of 1,4-diiodobutane, (•) concentration of tetrahydrothiophene (THT) prior to the addition of base; and (o) concentration of tetrahydrothiophene (THT) after the addition of base. Each point is the mean of three determinations.

12. The partially purified glutathione S-transferases retained the majority of the catalytic activity observed in the rat liver cytosol. Incubation of **1b** in a buffer containing GSH and glutathione S-transferases at 37°C resulted in the first-order disappearance of substrate (k 0.018 min^{-1}, $t_{\frac{1}{2}}$ 43.3 min). The time-course of disappearance of substrate and appearance of the product which gave tetrahydrothiophene on treatment with base are shown in Figure 1.

HPLC and TLC analysis of the mixture obtained after incubating **1b** with GSH and glutathione S-transferases indicated the presence of two major compounds, one with the same chromatographic mobility as GSH, and the second with the same mobility as **3**. Adjusting the pH of the mixture to $\geq$ 12 resulted in the formation of tetrahydrothiophene and the disappearance of the compound with the same mobility as **3** on HPLC (Fig. 2). The formation of tetrahydrothiophene was dependent on the concentration of the enzyme in the incubation

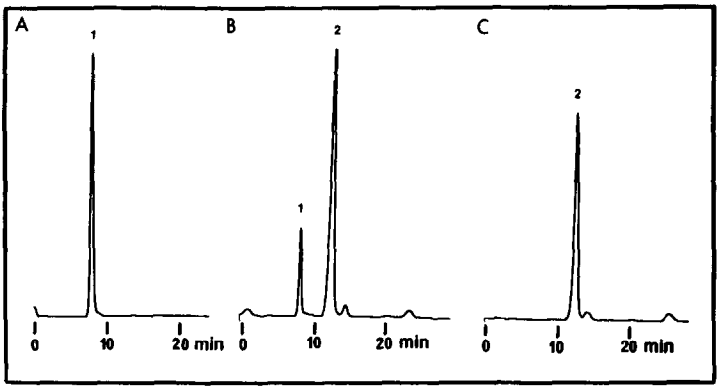


Figure 2. HPLC identification of Compound 3. Samples were analyzed by HPLC following the procedure outlined in the materials and methods section. A: An authentic sample of 3 (peak 1); B: Incubation mixture containing the product of the conjugation of 1b with GSH (peak 1) and GSH (peak 2); C: Incubation mixture after treatment with base.

mixture. Tetrahydrothiophene was not detected in incubation mixtures in which no enzyme or a boiled enzyme was added. These findings suggested that GLC analysis of tetrahydrothiophene produced by treatment of incubation mixtures with base is a reliable and convenient estimate of the amount of 3 formed and was used for this purpose in subsequent studies.

S-Benzylglutathione, a known inhibitor of glutathione S-transferases, was used to further confirm the involvement of these enzymes in the formation of 3. S-Benzylglutathione was a non-competitive inhibitor towards the substrate 1b (Fig. 3a) and a competitive inhibitor towards GSH (Fig. 3b). In absence of S-benzylglutathione the enzymatic reaction had a V_{max} of 0.53 (± 0.01) µmol/min/mg protein and Km of 0.31 (± 0.02) mM towards GSH, and a V_{max} of 0.46 (± 0.03) µmol/min/mg protein and Km of 0.24 (± 0.04) mM towards 1,4-diiodobutane.

The requirement for GSH as second substrate was also investigated. The enzyme preparation was passed through size exclusion column to remove GSH. The ability of the GSH free enzyme preparation to catalyze the conversion of 1b to 3 in presence of variable concentrations of GSH and cysteine, as an alternative sulfhydryl substrate, is shown in Fig. 4. The presence of a sulfhydryl compound is essential for activity. The addition of cysteine resulted

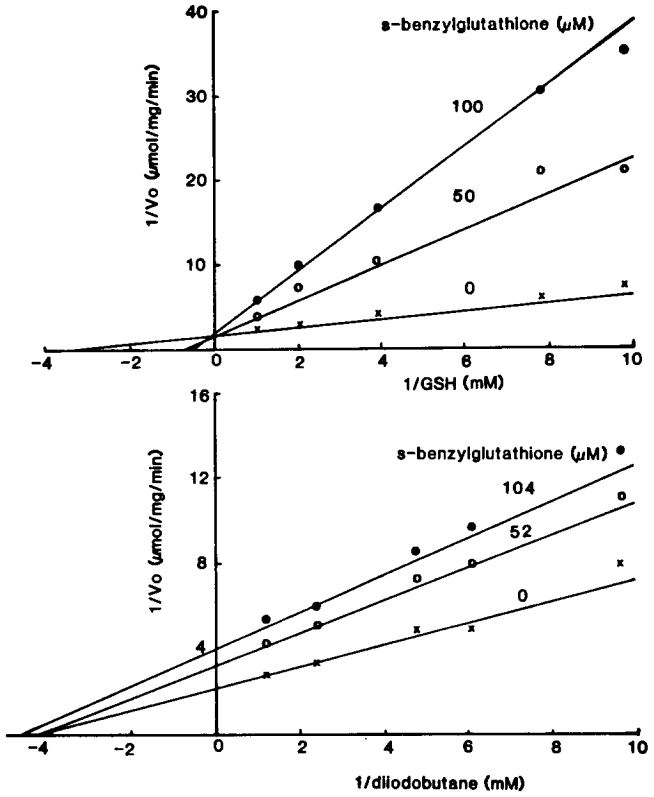


Figure 3. Effect of the addition of s-benzylglutathione on the conjugation of 1b. Incubations were conducted in a 0.1 M phosphate buffer (pH 7.4) containing glutathione S-transferase (0.034 mg/mL) at 37°C for 20 minutes. Incubation mixtures were treated as described under materials and methods. Incubation mixtures contained: Top. 1,4-diiodobutane (1.0 mM), varying concentrations of GSH and no S-benzylglutathione (x), 50 μM S-benzylglutathione (o), and 100 μM S-benzylglutathione (•). Bottom. Varying concentrations of 1,4-diiodobutane, GSH (1.0 mM) and no S-benzylglutathione (x), 52 μM S-benzylglutathione (o), or 104 μM S-benzylglutathione (•). Each point is the mean of three determinations.

in the recovery of about 5 percent of the activity observed in presence of GSH indicating that GSH is required for optimal enzyme activity.

CONCLUSION: The glutathione S-transferases catalyzed the conjugation of 1b with GSH *in vitro* to provide 3. The reaction followed saturation kinetics and its rate was dependent on the concentration of the substrate, the enzyme and GSH in the incubation mixture. However, it is not known as yet, if the glutathione S-transferases catalyze a similar reaction *in vivo*.

The formation of 3 involves two displacement reactions (Scheme 1). The first is a bimolecular reaction which is followed by an intramolecular cyclization. The first step appears to be catalyzed by the glutathione S-

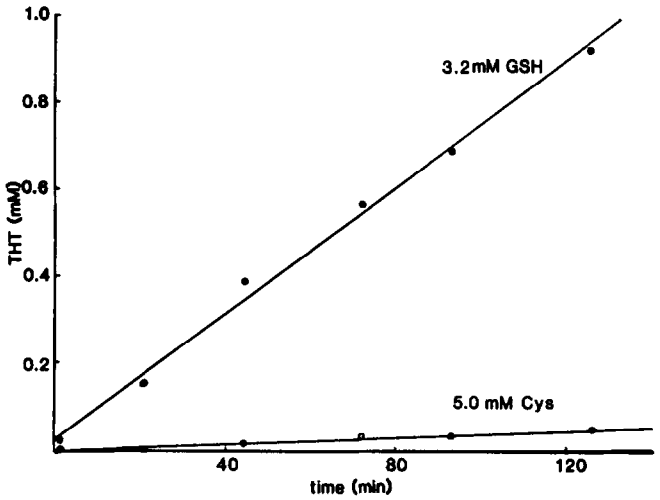
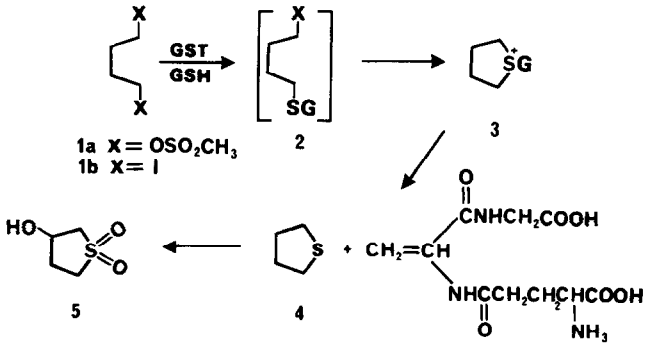


Figure 4. Effect of varying the concentration of GSH or cysteine on the conjugation of 1b. 1,4-Diiodobutane (1.03 mM) was incubated in 0.1 M phosphate buffer (pH 7.4) containing glutathione S-transferases (0.011 mg/mL) and 3.2 mM GSH (•) or 5.0 mM cysteine (o) at 37°C for 20 minutes. Incubation mixtures were treated as described under materials and methods for the determination of tetrahydrothiophene.

transferases. However, it is not known whether or not the intramolecular step also requires enzyme catalysis.

Recently, Buijo et al. (11) examined the direct mutagenic activity of a series of 18 α,ω-dihalogenoalkanes in Salmonella typhimurium strains TA 1530, TA 1535 and TA 100 using spot-test procedures and concluded that the mutagenicity of these compounds was dependent upon the nature of the halogen as well as the carbon chain length separating the two substituents. 1,4-Diiodobutane had the highest mutagenic activity in tests using the TA 1530



Scheme 1. Proposed pathway for metabolism of 1,4-disubstituted alkanes (GST, glutathione S-transferases).

and TA 1535 strains. The effects of conjugation with GSH on the mutagenic activity of 1,4-disubstituted butanes is not as yet known. Whether conjugation with GSH will result in increased or decreased mutagenic activity of these compounds will depend on the relative reactivity of 3 towards nucleophiles.

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