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# STEREOSELECTIVITY OF THE GLUTATHIONE S-TRANSFERASE CATALYZED CONJUGATION OF ARALKYL HALIDES

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SUMMARY: Rat cytosolic glutathione S-transferases catalyzed the conjugation of phenethyl chloride and phenethyl bromide with glutathione. The reaction proceeded with a high degree of stereoselectivity. The glutathione conjugate possessing the (R,S,S)- absolute configuration was formed in major quantities from the racemic substrates. The use of the enantiomers of the phenethyl chloride substrates indicated that the (S)-phenethyl chloride was conjugated in preference to the (R)-enantiomer. The conjugation proceeded with inversion of configuration at the benzylic carbon consistent with an $SN_2$-type mechanism. The stereoselectivity was greater for phenethyl chloride than for phenethyl bromide. Varying the substrate or enzyme concentration had no effect upon the observed stereoselectivity. The diastereomeric glutathione conjugates were separated by high performance liquid chromatography. These findings represent the first demonstration of the substrate stereoselectivity of the glutathione S-transferases.

INTRODUCTION: The glutathione S-transferases catalyze the conjugation of glutathione with an extremely broad variety of electrophilic, hydrophobic substrates including certain toxic xenobiotics (1,2). Catalysis appears to have no rigid specificity for either the carbon skeleton or the leaving group of the hydrophobic substrate (2). The normal mechanism for the conjugation appears to involve direct nucleophilic attack of the sulfhydryl group of glutathione at the electrophilic atom on the hydrophobic substrate (3,4). However, it has been suggested that at low glutathione concentrations, the catalysis may proceed via a double-displacement mechanism involving an enzyme-bound intermediate (3). The use of chiral substrates, in which the displacement reaction involves the chiral center, provides a straightforward means of determining the occurance of either a single or double displacement mechanism. The conjugation reaction between the chiral tripeptide, glutathione, and a racemic substrate would produce two diastereomers displaying

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different physical properties. Further, reaction of glutathione with one of the substrate enantiomers should produce only one of these diastereomers, if the reaction is not accompanied by racemization at the chiral center. Establishing the absolute configuration of the product would then indicate whether the reaction proceeded with retention or inversion of configuration at the chiral center, thereby distinguishing between the two mechanisms.

In our mechanistic studies of glutathione S-transferase catalysis using chiral substrates, we have unexpectedly encountered a high degree of substrate stereoselectivity. This stereoselectivity is particularly unusual and unexpected considering the very large variety of substrates that can undergo catalysis with these isozymes.

MATERIALS AND METHODS: Optically pure (R) and (S)-mandelic acids were purchased from Aldrich Chemical Company. Reduced glutathione was obtained from Sigma Chemical Company.

Racemic phenethyl chloride was prepared from 1-phenylethanol using $POCl_3$ by the method of Siegel and Graefe (5). Racemic phenethyl bromide was obtained from 1-phenylethanol using $PBr_3$ by the method of Rupe and Tomi (6).

Synthesis of Optically Active Substrates from Optically Pure Mandelic Acids (8). Mandelic acid was reduced with borane-THF** complex to the corresponding phenylethylene glycol using the procedure of Yoon et al. (7). Phenylethylene glycol was converted to 1-phenylethanol by the procedure of Maylin et al. (8). The chiral 1-phenylethanol was converted to the corresponding chloro compound using $POCl_3$ by the method of Siegel and Graefe (5). (S)-isomer, 52% yield $[\alpha]_D^{24}$ -99.3° (neat), 78.8% e.e., Anal. ($C_8H_9Cl$); Calcd: C 68.34, H 6.45, Cl 25.21; Found: C 68.20, H 6.86, Cl 24.74; (R)-isomer, 46% yield, $[\alpha]_D^{23}$ + 99.6° (neat), 79.0% e.e., Anal. ($C_8H_9Cl$); C 68.34, H, 6.45, Cl 25.21; Found: C 69.02, H 6.78, Cl 23.96. The optical purity of the chiral phenethyl chlorides was verified by reaction with glutathione (as described below) to form the corresponding S-(1-phenylethyl)-glutathiones. The diasteriomeric purities of the conjugates obtained from the (S)-chloro and (R)-chloro compounds were 76.2% and 81.4%, respectively.

S-(1-Phenylethyl)-glutathione. Glutathione (10 mmol, 3.07 g) was dissolved in 1.2 M KOH (25 mL). Ethanol (25 mL) was added followed by (R,S)-phenethyl chloride (10 mmol, 1.40 g) and a catalytic amount of 18-Crown-6 (1 mmol, 264 mg) (Aldrich Chemical Company). The reaction was stirred (3 h) and then adjusted to pH 3.2 (conc. HCl), concentrated and extracted with diethyl ether. Solids formed during concentration or extraction were isolated by filtration. The aqueous layer was evaporated to dryness. The combined solids were recrystallized from hot water. Product enriched in the (S,S,S)-diastereomer crystallized first; Anal. ($C_{18}H_{26}N_3O_6S$); Calcd; C 52.54, H, 6.12, S 7.93; Found: 52.62, H 6.08, S 8.05. The mother liquor provided product enriched in the (R,S,S)-diastereomer

** Abbreviations: HPLC, high performance liquid chromatography; THF, tetrahydrofuran, TCA, trichloroacetic acid; e.e., enantiomeric excess; EDTA, ethylenediaminetetraacetic acid; and GSH, glutathione.

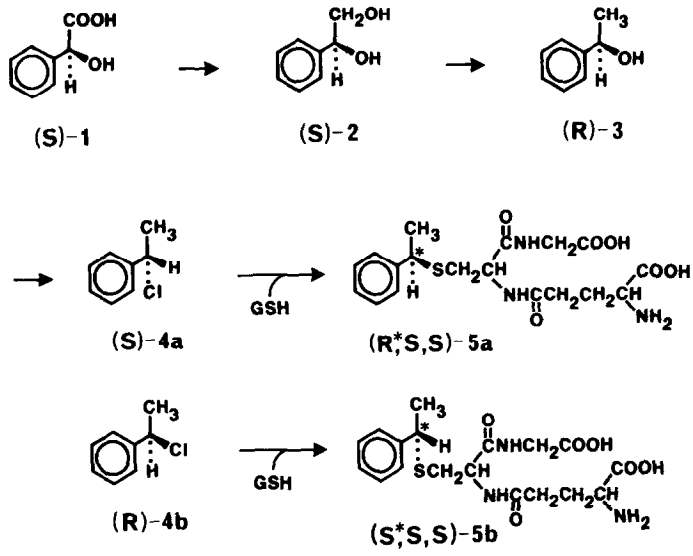
upon concentration; Anal. ($C_{18}H_{26}N_3O_6S$); Calcd: C 52.54, H 6.12, S 7.93; Found: C 52.36, H 6.16, S 7.94. Isolated yields were generally about 60%.

Enzyme Preparation. A 20% liver homogenate in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA was obtained from male Sprague-Dawley rats (150-200 g). The 105,000 g supernatant was used as the source of the glutathione S-transferase activity. Protein was measured by a dye-binding assay (Bio-Rad Protein Assay Kit; Bio-Rad Laboratories), using bovine serum albumin as the standard.

Enzyme Incubations. Enzyme incubations were performed at pH 7.5 in 0.1 M potassium phosphate buffer at 25° C for 5 minutes. Standard incubation mixtures contained 15.2 mg of protein (as 105,000 g liver supernatant), 5 mM glutathione, 2 mM substrate, 1 mM EDTA and 4% (v/v) ethanol in a total volume of 10 mL. The incubations were terminated by the addition of 20% TCA (2 mL). The precipitated protein was removed by centrifugation and the supernatant was extracted with methylene chloride. The methylene chloride layer was discarded and the aqueous layer was adjusted to pH 5 by the dropwise addition of 4.6 M sodium acetate. The aqueous solution was extracted with methylene chloride and a 9 mL aliquot of the aqueous layer was passed through a 1 x 4 cm Amberlite$^R$ XAD-4 resin column. A stepwise methanol-water gradient was used to elute the glutathione conjugates. The 20-40% fractions were combined and concentrated to dryness under reduced pressure. The resulting residue was reconstituted with 100 µL of distilled water and an aliquot was analyzed by HPLC. Quantitation of the conjugates was carried out by measuring chromatographic peak heights. The concentrations of the conjugates in the original incubation mixtures were calculated by comparing their chromatographic peak heights with those obtained from known amounts of authentic samples of the conjugates added to the incubation mixtures. Recoveries of the two diastereomers from the incubation mixture were determined using synthetically prepared glutathione conjugates. At the concentrations tested, the two diastereomers were recovered equally from the incubation mixture.

RESULTS AND DISCUSSION: The chiral substrates were synthesized from optically pure mandelic acids of known absolute configuration. The sequential reduction of the carboxyl group conserved the configurational integrity of the benzylic carbon. The resulting chiral benzylic alcohols were converted to the corresponding benzylic halides by reactions which proceed primarily with inversion of configuration with some racemization (5, 9). The reaction of phenethyl halides with sulfhydryl nucleophiles has been demonstrated to proceed with inversion of configuration consistent with an $SN_2$-type mechanism (5, 10). Thus, the assignment of the absolute configuration of the resulting glutathione adducts was based upon the known absolute configuration of the starting halides. The stereochemical course of a typical synthetic sequence is illustrated in Scheme I.

The diasteriomeric glutathione conjugates were separated using HPLC. Figure 1 represents the HPLC separation of the two diastereomers obtained from the reaction of glutathione with the different substrates.




Scheme I. Stereochemical course of the formation of the diastereomeric glutathione conjugates from optically active mandelic acids. The absolute configurations of the specific intermediates are designated in parentheses below the structures.

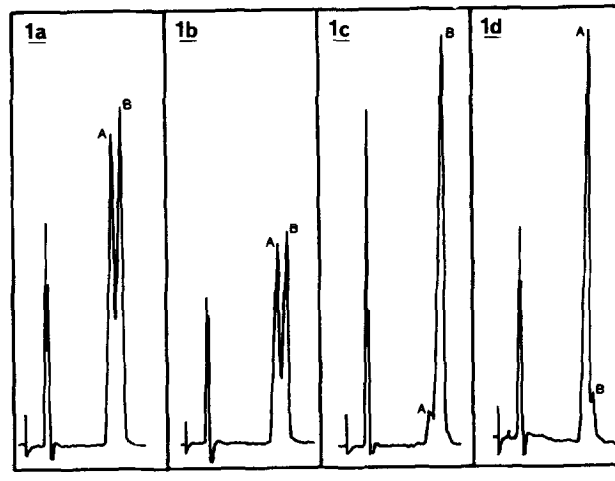



Fig. 1. HPLC separation of the diastereomeric glutathione conjugates formed by reaction of glutathione with: (a) (R,S)-phenethyl bromide, (b) (R,S)-phenethyl chloride, (c) (R)-phenethyl chloride and (d) (S)-phenethyl chloride. Peak A corresponds to the diastereomer having the (R,S,S)-configuration and peak B to the diastereomer of the (S,S,S)-configuration. The analysis was performed on a μ-Bondapak C-18 column (Waters Associates; Milford, Mass.) using 12% isopropyl alcohol in 0.01 M acetic acid as the mobile phase at a flow rate of 1.5 mL/min and a UV detector set at 254 nm.

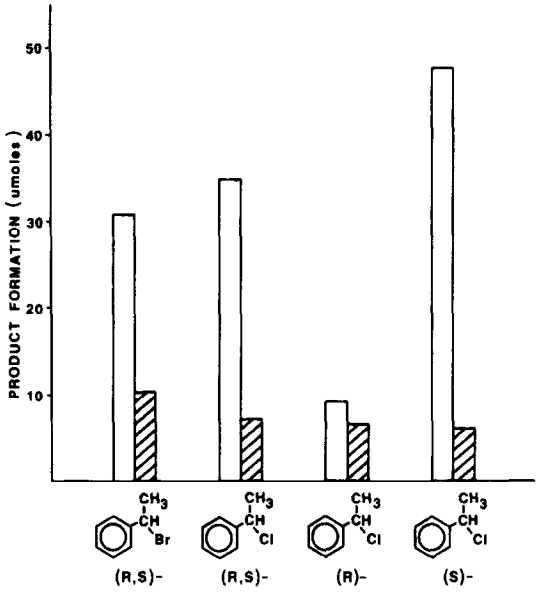



Fig. 2. Enzymatic formation of glutathione conjugates from phenethyl bromide, phenethyl chloride and its enantiomers. The open bars represent the product having the (R,S,S)-configuration and the cross-hatched bars correspond to the product with the (S,S,S)-configuration. Each point represents the average of two separate experiments.

Incubation of (R,S)-phenethyl chloride with the 105,000 g supernatant of rat liver in the presence of 5 mM glutathione resulted in the formation of the glutathione conjugates 5a and 5b. These thioethers were not produced in equal amounts as expected. The ratio of the (R,S,S)-diastereomer (5a) to that of the (S,S,S)-diastereomer (5b) was about 5 (Fig. 2). Incubation of the (R,S)-phenethyl bromide under the same reaction conditions also resulted in the formation of a mixture of 5a and 5b, but the ratio (5a/5b) was only 3. The use of enantiomerically enriched substrates established the origin of the preferential formation of 5a from the racemic substrates. Incubation of phenethyl chloride enriched with the (S)-enantiomer resulted in higher yields of the glutathione conjugates than those obtained from an equimolar amount of the racemate. The ratio (5a/5b) in the product was approximately 8. In contrast, the incubation of the substrate enriched in the (R)-enantiomer resulted in a much lower yield of the conjugates and the ratio (5a/5b) in the product was about 1.4. These experiments suggested

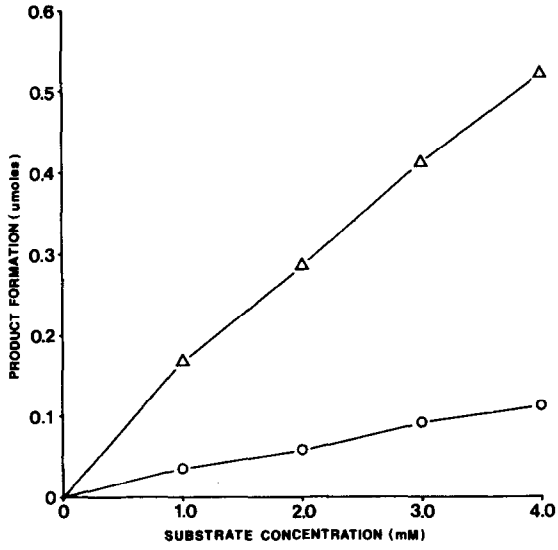



Fig. 3. Effect of varying the substrate concentration on the enzymatic conjugation of racemic phenethyl chloride with glutathione. The triangles designate the product having the (R,S,S)-configuration and the circles represent the product of the (S,S,S)-configuration. Each point represents the average of two separate experiments.

that these conjugation reactions proceeded with a high degree of stereoselectivity. The (S)-enantiomer was a better substrate than the (R)-enantiomer for the rat liver glutathione S-transferase activities. Furthermore, an examination of the stereochemical course of the conjugation reaction between the substrate enriched in the (S)-enantiomer and glutathione indicated that the reaction proceeded with an inversion of configuration at the benzylic carbon of the substrate. This observation is consistent with the $SN_2$ mechanism proposed for the conjugation reactions in the presence of high concentrations of glutathione (3).

To further verify that the stereoselectivity phenomenon was enzymatic in nature, the effect of varying the substrate concentration (Fig. 3) and the protein concentration (Fig. 4) on the amount of conjugate formation and the relative ratios of the diastereomers was determined. It was found that the total conjugate formation was proportional to both the substrate concentration as well as to the amount of added protein (enzyme). The ratio (5a/5b) of the diasteriomers remained constant in all cases when

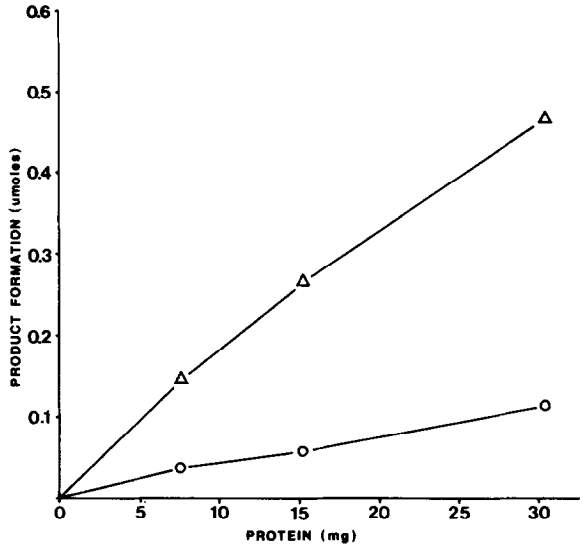



Fig. 4. Effect of varying the protein concentration on the enzymatic conjugation of racemic phenethyl chloride with glutathione. The triangles represent the product of the (R,S,S)-configuration and the circles correspond to the product of the (S,S,S)-configuration. Each point represents the average of two separate experiments.

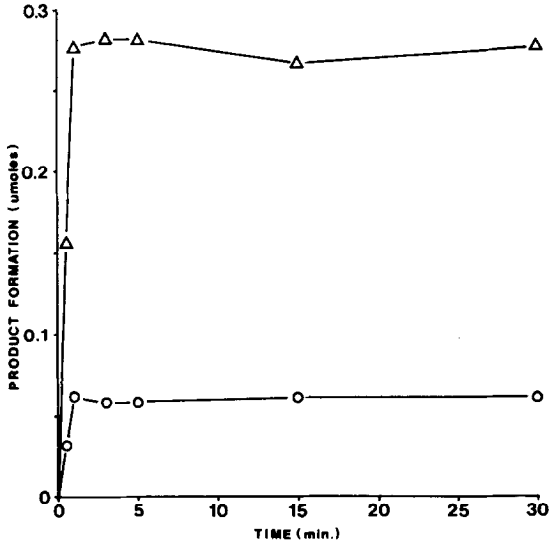



Fig. 5. Time course of the enzymatic conjugation of racemic phenethyl chloride with glutathione. The triangles represent the product of the (R,S,S)-configuration and the circles correspond to the product of the (S,S,S)-configuration. Each point represents the average of two separate experiments except at 30 sec which was a single experiment.

the racemic phenethyl chloride substrate was tested. The time course of the enzymatic reaction was studied and the reaction was found to be rapid proceeding maximally in about five minutes (Fig. 5). When either boiled enzyme or no enzyme was used in the incubation mixture, the formation of conjugate was not detected.

The observation of stereoselectivity in the case of the glutathione S-transferases should not be viewed as an anomoly. In fact, enzyme catalysis commonly demonstrates rigid and absolute stereospecificity with few exceptions. Enantiomeric preference is not unreasonable since enzymes are essentially chiral reagents (being composed of (S)-amino acids). Generally, the exceptions to the observed rigid stereospecificity have been the xenobiotic metabolizing enzymes. The lesser degree of specificity exhibited by these enzymes has been attributed to the need to metabolize a large variety of different substrates. However, certain metabolizing enzymes do exhibit stereochemical preferences for some substrates (11).

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