Stereochemical Aspects of Conjugation Reactions Catalyzed by Rat Liver Glutathione *S*-Transferase Isozymes

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Substrate enantioselectivity in the conjugation of phenethyl halides catalyzed by the glutathione *S*-transferases was studied with partially purified isozymes from rat liver. All of the isozymes tested possessed measurable activity with phenethyl chloride. Tranferase A was the most active isozyme tested. Each of the isozymes demonstrated a high degree of substrate enantioselectivity, with transferase A being the most enantioselective isozyme. The enantioselectivity was determined by high-pressure liquid chromatographic analysis of the enzymatically formed diastereomeric products. The effect of limiting glutathione concentrations on the stereochemical outcome of the transferase A catalyzed conjugation of the chiral substrate, (*S*)-phenethyl chloride (4 mM), was investigated. The stereochemical course of the enzymatic reaction was not significantly altered at glutathione concentrations as low as 25 μM. The major product of conjugation had the opposite stereochemistry at the benzylic carbon, indicating that the reaction proceeded primarily with inversion of configuration. The glutathione conjugates, *S*-[(*R*)-1-phenylethyl]glutathione, *S*-[(*S*)-1-phenylethyl]glutathione, *S*-benzylglutathione, and *S*-methylglutathione were studied as inhibitors of the transferase A catalyzed conjugation of 1-chloro-2,4-dinitrobenzene. The order of the inhibitory potency was *S*-[(*S*)-1-phenylethyl]glutathione = *S*-benzylglutathione > *S*-[(*R*)-1-phenylethyl]glutathione > *S*-methylglutathione. This represented the first demonstration of the stereoselective product inhibition of the glutathione *S*-transferases.

The glutathione *S*-transferases (EC 2.5.1.18) are cytosolic enzymes that are important in the modulation of the toxicity of endogenous and exogenous hydrophobic compounds.[1] The generally accepted mechanism of catalysis of these enzymes is a single displacement mechanism that involves the direct nucleophilic attack of the activated sulfhydryl group of glutathione on the electrophilic center of the hydrophobic substrate.[2] However, three recent observations have suggested that a double-displacement mechanism, involving a covalently bound enzyme–substrate intermediate, may be operating in the conjugation of certain substrates, especially in the presence of low glutathione concentrations. First, the kinetics of the conjugation of 1-chloro-2,4-dinitrobenzene catalyzed by rat liver transferase A were complex and produced a biphasic double-reciprocal plot of velocity vs. glutathione concentration.[3,4] These complex kinetics have led to two proposals. Mannervik et al. suggested that the reaction proceeds by a random sequential mechanism and attributed the complex kinetics to product inhibition.[5-7] On the other hand, Jakoby et al. proposed a hybrid bibi–ping-pong mechanism.[3] At high concentrations of glutathione, such as those normally found in rat liver, the reaction proceeds by a single displacement mechanism involving an ordered bibi process. At limiting glutathione concentrations, the ping-pong mechanism would be operable. The second observation, which supports the notion that an enzyme-bound intermediate is formed during catalysis, is the demonstration that a catalytically essential sulfhydryl group is presnet at the hydrophobic binding site of transferase A.[3,8,9] Finally, it has been observed that glutathione *S*-transferases can form covalently bound complexes with a number of substrates, such as aminoazo-dye carcinogens, benzyl chloride, and 1-chloro-2,4-dinitrobenzene.[10,11] These observations, however, are not sufficient to establish the formation of enzyme-bound intermediates. The study of the stereochemical course of the reactions catalyzed by the transferases with chiral substrates in which the chiral center is involved in the conjugation reaction should provide unequivocal evidence of the occurrence of either a single- or double-displacement mechanism. If the reaction involves a single or odd number of displacements, inversion of configuration at the reaction site would be observed. On the other hand, retention of configuration would indicate double or even-number displacements.

Recently, we began an investigation of the stereochemical aspects of a variety of conjugation reactions catalyzed by the glutathione *S*-transferases. In these studies, we utilized chiral model compounds of known absolute configuration as substrates and determined the absolute configuration of the products. In preliminary studies, we observed that rat cytosolic glutathione *S*-transferases catalyzed the conjugation of (±)-1-chloro-1-phenylethane (**3**) and (±)-1-bromo-1-phenylethane with glutathione to provide the conjugates **4** and **5**.[12] However, the thioethers **4** and **5** were not formed in equal amounts in the products. The ratio of **4** to **5** in the product obtained from (±)-**3** in the presence of 5 mM glutathione was 4.9. This suggested that the reaction proceeded either with substrate enantioselectivity, i.e., the enzyme preferentially catalyzed the conjugation of one of the enantiomers of the racemic substrate, or with product stereoselectivity, i.e., the two enantiomers of the substrate were conjugated equally well but one of the diastereomeric products was formed preferentially. One way to differentiate between these two possibilities is for one to study the conjugation reaction using the enantiomers of **3** as substrates. Unfortunately,

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(10) Ketterer, B.; Tipping, E.; Beale, D.; Meuwissen, J. A. T. P. In ref 2; pp 243–253.
(11) Ketterer, B.; Christodoulides, L. *Chem.-Biol. Interact.* **1969**, *1*, 173.
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0022-2623/83/1826-0066$01.50/0

it was not possible to obtain the optically pure enantiomers of **3** due to the facile racemization of the chiral benzylic carbon. Consequently, these reactions were studied with enantiomerically enriched substrates. Incubation of enantiomerically enriched (*S*)-**3** with liver cytosol in the presence of glutathione (5 mM) resulted in the formation of higher yields of the conjugates than those obtained from an equimolar concentration of the racemate. In contrast, the incubation of the substrate enriched in (*R*)-**3** resulted in much lower yields of the conjugates. These results indicated that the conjugation reactions proceeded with substrate enantioselectivity and that the *S* enantiomer was a better substrate than the *R* enantiomer. Furthermore, the ratio of **4** to **5** in the product obtained from (*S*)-**3** was 7.6 and was higher than that obtained with (±)-**3**. The absolute configuration of the benzylic carbon in the major diastereomer **4** was opposite to that of the predominant enantiomer in the substrate. This indicated that the conjugation reaction, in the presence of a high concentration of glutathione (5 mM), proceeded primarily with inversion of configuration at the benzylic carbon. This was further confirmed by the observation that the ratio of **5** to **4** in the product formed from the enantiomerically enriched (*R*)-**3** substrate was 0.7 and was greater than that obtained form (±)-**3** (0.2).

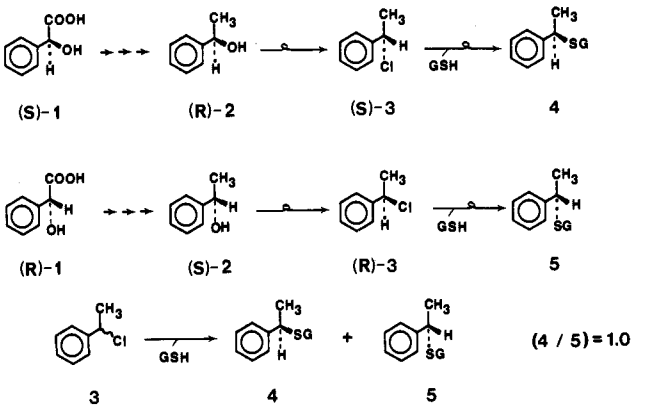
Interestingly, the ratio of **4** to **5** in the product obtained with enantiomerically enriched (*S*)-**3** was only 7.6. This value is much lower than the predicted value of 19.6, which could be calculated by multiplying the ratio obtained when (±)-**3** was used as substrate (4.9) by the enantiomeric ratio of the substrate (4.0). However, enantiomerically enriched (*R*)-**3** gave products in which the ratio of **4** to **5** was 1.4 and was close to the calculated value of 1.2.

In this paper we describe some additional studies on the stereochemical aspects of the conjugation of **3** with glutathione. Since the conjugation of **3** appeared to proceed with inversion of configuration in the presence of high glutathione concentration (5 mM),[12] it was of interest to determine if lowering the glutathione concentration will cause the enzymatic conjugation reaction to proceed with retention of configuration, as would be predicted from the double-displacement mechanism proposed by Jakoby et al.[3] Secondly, the crude preparation of the glutathione *S*-transferases exhibited a high degree of substrate enantioselectivity.[12] Since the cytosolic fraction contains several transferase isozymes, it was not clear whether each of the transferases exhibited the same degree of substrate enantioselectivity or not. Therefore, we examined the substrate enantioselectivity of the partially purified rat liver glutathione *S*-transferase isozymes with (±)-**3** as substrate. Finally, we examined the inhibitory effects of the diastereomers *S*-[(*R*)-1-phenylethyl]glutathione (**4**) and *S*-[(*S*)-1-phenylethyl]glutathione (**5**), the products of the enzymatic conjugation of (*S*)-**3** and (*R*)-**3**, respectively, on the enzymatic conjugation of 1-chloro-2,4-dinitrobenzene catalyzed by glutathione *S*-transferase A.

Results and Discussion

Chemical Synthesis and Assignment of Absolute Stereochemistry. The synthesis of the enantiomerically enriched phenethyl chlorides, (*R*)-**3** and (*S*)-**3** and the glutathione conjugates **4** and **5** are summarized in Scheme I. Reduction of the carboxy group of optically pure mandelic acids of known absolute configuration by established procedures provided high yields of the corresponding chiral 1-phenylethanols. Halogenation of the optically active alcohols proceeds by an inversion of configuration with some racemization. Thus, treatment of (*R*)-(+)-**2** with phosphorus oxychloride and pyridine provided moderate yields of chemically pure **3**. Measurements of the optical rotation of the product indicated that it was composed of 79.8% of (*S*)-(−)-**3** and 20.2% of (*R*)-(+)-**3**. Similarly, treatment of (*S*)-(−)-**2** with phosphorus oxychloride and pyridine provided a product that contained 79.0% of (*R*)-(+)-**3** and 21.0% (*S*)-(−)-**3**. The enantiomeric composition of (*R*)-(+)-**3** and (*S*)-(−)-**3** was confirmed by the nonenzymatic reaction with glutathione. The nonenzymatic reactions of glutathione with (*R*)-(+)-**3** produced a mixture of **4** and **5** in the ratio of 1:4. Similarly, the reaction of glutathione with (*S*)-(−)-**3** produced a mixture of **4** and **5** in the ratio of 4:1. Since the nonenzymatic displacement of the chloride ion in **3** with the thiolate anion of glutathione proceeds with inversion of configuration,[13] the absolute configuration of the benzylic carbons in **4** and **5** are assigned as *R* and *S*, respectively.[12]

Scheme I. Stereochemical Course of the Synthetic Sequence Used for the Preparation of the Enantiomerically Enriched Substrates (*S*)-**3** and (*R*)-**3** and Diastereomeric Glutathione Conjugates **4** and **5**



The nonenzymatic reaction of (±)-**3** with glutathione provided *S*-(1-phenylethyl)glutathione. The diastereomers of *S*-(1-phenylethyl)glutathione (**4** and **5**) were separable by HPLC.[12] Fractional recrystallization of the product obtained from the reaction of (±)-**3** with glutathione provided products that were enriched with either **4** or **5**. Chemically pure samples of *S*-(1-phenylethyl)glutathione that contained 95% of **5** and 5% of **4** or 70% of **4** and 30% of **5** were obtained.

Substrate Enantioselectivity of the Glutathione *S*-Transferase Isozymes. The glutathione *S*-transferase isozymes were separated from the rat liver cytosols by a modification of the method reported by Habig et al.[14] The isozymes were purified through the carboxymethylcellulose step, and a summary of a typical purification is given in Table I. The elution order was used to distinguish the isozymes as described by Habig et al.[14] We determined the relative rate of conjugation of (±)-**3** by the different rat liver isozymes by measuring the total amount of conjugates (**4** and **5**) formed after incubation for 5 min using standard incubation conditions. We determined the enantioselectivity of each of the isozymes by calculating the ratio of diastereomers (**4**/**5**) in the product.

Each of the isozymes tested possessed measurable activity for the substrate (±)-**3** in keeping with the broadly overlapping nature of their substrate specificities (Table II). Transferase A, as expected, possessed the highest specific activity for this substrate. The contribution of

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Table I. Summary of the Purification of Rat Liver Glutathione S-Transferase Isozymes

purification step	total protein,[a] mg	total act.,[b] μmol min^{-1}	sp act., μmol min^{-1} mg^{-1}
crude extract	10 700	2140	0.2
DEAE-cellulose	840	1882	2.2
CM-cellulose			
AA	10	31 (3)[c]	3.1
A	14	170 (16)	12.0
B	32	302 (28)	9.6
C	38	98 (9)	2.6
rinse[d]	180	486 (45)	2.7
total	274	1088 (100)	

[a] Protein was determined by a dye-binding assay [Bradford, M. M. *Anal. Biochem.* 1976, *72*, 248–254]. [b] We determined the GSH S-transferase activity by measuring the initial rate of enzymatic conjugation of 1-chloro-2,4-dinitrobenzene. [c] Percent of total activity eluted from the CM-cellulose column. [d] Rinse contains transferases D and E, which are not retained on the carboxymethylcellulose column and elute with the buffer rinse in the first 20–30 fractions.

Table II. Specific Activity and Substrate Enantioselectivity of Rat Liver Glutathione S-Transferase Isozymes

	isozyme				
	AA	A	B	C	rinse
total act.,[a] nmol 5 min^{-1}	117	3209	1588	650	414
specific act., nmol 5 min^{-1} mg^{-1}	12	226	50	17	2
% of total act.	2	54	27	11	7
ratio of 4 to 5	*b*	5.9	4.4	4.2	4.4

[a] The substrate (±)-phenethyl chloride (4 mM) was incubated for 5 min with the enzyme in the presence of glutathione (5 mM). The amount of product found was determined by HPLC. Total activity refers to the total amount of conjugates (4 and 5) found. [b] The amount of conjugate formed was too small to permit accurate determination of the ratio of 4 to 5 in the product.

each of the isozymes to the conjugation of (±)-**3** was calculated from the specific activity and the total amount of protein for each of the isozymes (Table II). Transferase A accounted for approximately half of the conjugative activity recovered from the carboxymethylcellulose column.

All of the isozymes exhibited a high degree of substrate enantioselectivity (Table II). Transferase A, the most active isozyme toward this substrate, proved to be the most enantioselective as well. The similarity in enantioselectivity of these isozymes may have mechanistic significance and is perhaps an indication of intrinsic similarities in their catalytic active sites.

Stereochemical Course of Transferase A Catalyzed Conjugation of (*S*)-(−)-3 in the Presence of Low Concentrations of Glutathione. The conjugation of enantiomerically enriched (*S*)-(−)-**3** (80% ee) catalyzed by rat liver glutathione S-transferase A in the presence of 60, 53, 35, 25, and 10 μM glutathione was investigated. A mixture of the diastereomers **4** and **5** was produced. The diastereomeric conjugate **4** was invariably the major product obtained from (*S*)-(−)-**3**. The ratio of **4** to **5** in the product ranged from 5.1 to 5.6 at glutathione concentrations of 25–60 μM, respectively. The amount of product formed in the presence of 10 μM glutathione was too small to allow accurate estimation of the ratio **4**/**5**. Interestingly, the highest ratio of **4** to **5** in the product under these conditions was only 5.6. In the presence of 5 mM glutathione, the enzymatic conjugation of (*S*)-(−)-**3** by rat liver cytosol produced a product in which the ratio of **4** to **5** was 7.7.[12] The reasons for the decreased ratio of **4** to **5** in the enzymatic conjugation catalyzed by glutathione S-transferase A in the presence of low concentrations of glutathione (25–60 μM) are not clear. However, it should be pointed out that the kinetics of the reactions catalyzed by these enzymes are complex and depend on several factors, including the relative concentrations of the hydrophobic substrate, glutathione, and products. Therefore, it is not possible to extrapolate from one set of reaction conditions to another.

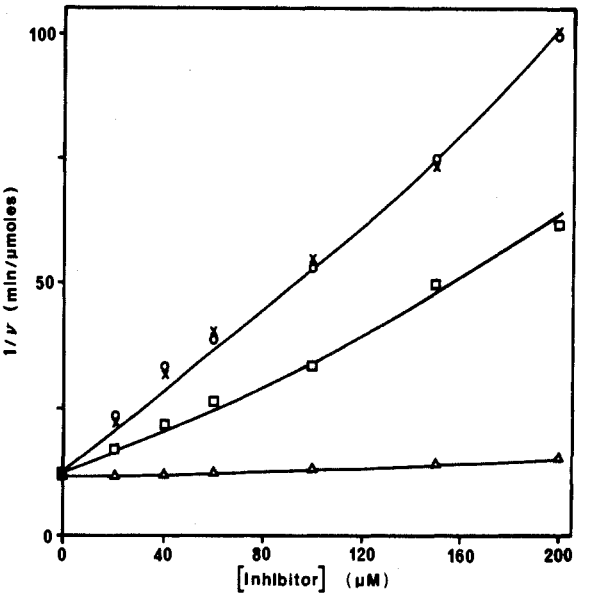

Figure 1. Inhibition by *S*-[(*R*)-1-phenylethyl]glutathione (○), *S*-benzylglutathione (X), *S*-[(*S*)-1-phenylethyl]glutathione (□), and S-methylglutathione (△) of the glutathione S-transferase A catalyzed conjugation of 1-chloro-2,4-dinitrobenzene (0.2 mM). Glutathione concentration was 0.5 mM.

The absolute configuration of the benzylic carbon of the major diastereomer (**4**) in the product of conjugation of (*S*)-(−)-**3** was opposite to that of the substrate. This indicates that the reaction proceeded primarily with inversion of configuration at the benzylic carbon. Changing the glutathione concentration from 5 mM to 25 μM did not produce a significant change in the stereochemical course of reaction. This suggested that the mechanism of catalysis was not changed by limiting the glutathione concentration and involved a direct nucleophilic attack of the sulfhydryl group on the electrophilic carbon. These results do not support the involvement of an enzyme-bound intermediate or a double-displacement mechanism at low glutathione concentration. The occurrence of such an intermediate cannot be ruled out for other substrates, however.

Inhibition by S-Substituted Glutathione Analogues of the Conjugation of 1-Chloro-2,4-dinitrobenzene Catalyzed by Glutathione *S*-Transferase A. Conjugates **4** and **5** were both potent inhibitors of the transferase A catalyzed conjugation of 1-chloro-2,4-dinitrobenzene with glutathione (Figure 1). *S*-Methylglutathione and *S*-benzylglutathione are known inhibitors of this enzymatic conjugation reaction and, therefore, were studied to determine the relative inhibitory potencies of **4** and **5**. The inhibitory activities of these glutathione conjugates were calculated from Dixon plots. The relative order of potency (as indicated by either slope or I_{50} values) was found to be as follows: **5** = *S*-benzylglutathione > **4** > *S*-methylglutathione (Figure 1). The kinetics of inhibition were nonlinear at high inhibitor concentration, similar to that

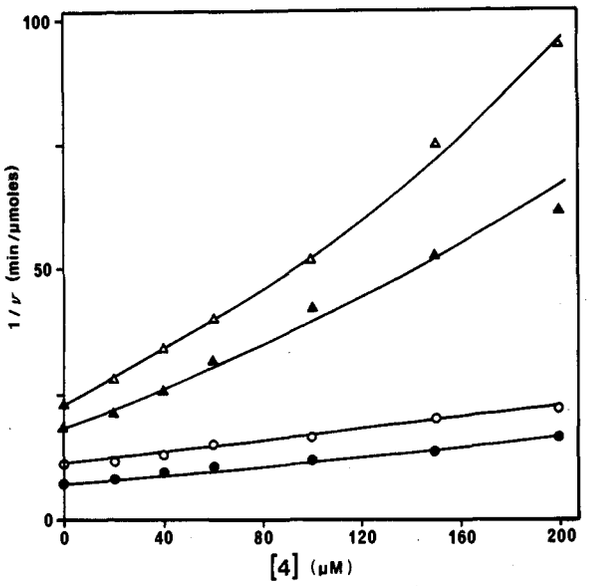

Figure 2. Inhibition by *S*-[(*R*)-1-phenylethyl]glutathione of the glutathione *S*-transferase A catalyzed conjugation of 1-chloro-2,4-dinitrobenzene. The concentrations of 1-chloro-2,4-dinitrobenzene and glutathione were, respectively, 0.2 and 0.5 (△), 0.7 and 0.5 (▲), 0.2 and 10.0 (○), and 0.7 and 10.0 (●) mM.

reported for other S-substituted glutathione derivatives.[9,15] The inhibition by **4** and **5** appeared to be competitive with glutathione and noncompetitive with 1-chloro-2,4-dinitrobenzene when the glutathione concentration was 10 mM (Figures 2 and 3). However, the inhibitory kinetics were more complex at a glutathione concentration of 0.5 mM.

The inhibitory activity of various S-substituted glutathione analogues was reported to be related to the hydrophobicities of the S substituents.[9] The conjugates **4** and **5** are diastereomers that display different physiochemical properties, and the difference in inhibitory activity between **4** and **5** may be due to the differences in their hydrophobicity. We determined the relative hydrophobicities of **4** and **5** by measuring their retention volumes (k') on a bonded octadecylsilane column using high-pressure liquid chromatography. Previously, it has been shown that column retention values correlate well with the lipid–water partition values determined by other methods.[16,17] The order of hydrophobicity of these compounds was as follows: **5** > **4** > *S*-benzylglutathione. Therefore, the differences in hydrophobicity alone could not account for the differences in the inhibitory activities of these compounds. *S*-Benzylglutathione, which had the lowest retention volume, was much more active an inhibitor than **4** and was equipotent to **5**.

The difference in inhibitory activities of the substituted glutathione derivatives is most likely a reflection of the difference in their binding affinities to the active site. The kinetics of inhibition by several S-substituted glutathione analogues have suggested that these derivatives compete for the glutathione binding site.[3,6,15] The kinetics obtained with **4** and **5** as inhibitors provide qualitatively similar results to those reported for other S-substituted glutathione derivatives. Therefore, given a common interaction

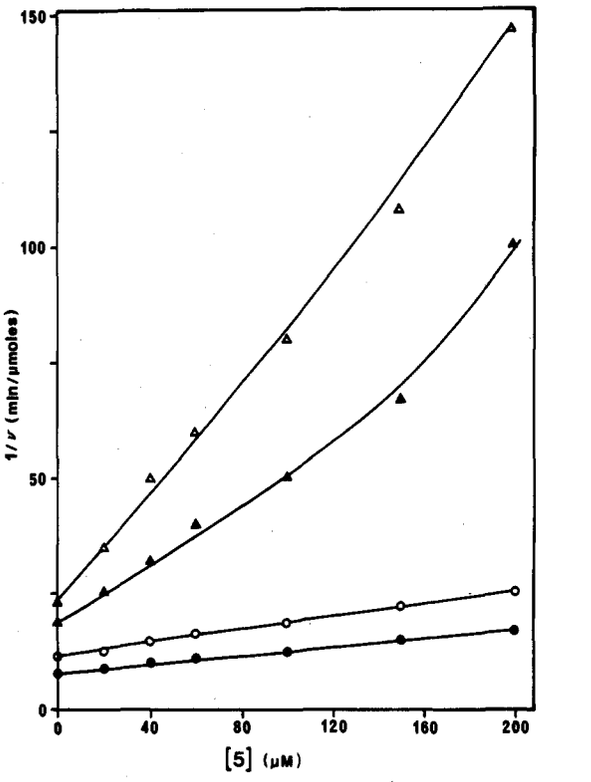

Figure 3. Inhibition by *S*-[(*S*)-1-phenylethyl]glutathione of the glutathione *S*-transferase A catalyzed conjugation of 1-chloro-2,4-dinitrobenzene. The concentrations of 1-chloro-2,4-dinitrobenzene and glutathione were, respectively, 0.2 and 0.5 (△), 0.7 and 0.5 (▲), 0.2 and 10.0 (○), and 0.7 and 10.0 (●) mM.

with the glutathione binding site, differences in inhibitory activity may reflect affinity difference due to the stereochemistry of the phenylethyl substituent. Regardless of its origin, the differences in inhibitory potencies of **4** and **5** represent the first demonstration of the stereoselective product inhibition of the glutathione *S*-transferases.

The origin of the enantioselective enzymatic conjugation of **3** can be explained based upon the differences in the inhibitory activities of **4** and **5**. Compounds **4** and **5** are the products of the nucleophilic substitution reaction of glutathione with the enantiomers (*S*)-**3** and (*R*)-**3**, respectively. One possibility is that the product release from the enzyme–product complex is rate limiting in the transferase-catalyzed conjugation of the enantiomers, (*S*)-**3** and (*R*)-**3**. The higher affinity of **5**, the product of glutathione conjugation with (*R*)-**3**, would result in its slower release from the enzyme–product complex, which would be reflected in the slower overall rate of conjugation of (*R*)-**3**. The origin of the stereoselectivity could also be explained based upon possible differences in the binding affinities of the substrates, (*R*)-**3** and (*S*)-**3**. As indicated earlier, the higher affinity of **5** for the enzyme is most likely attributed to the favorable orientation of the substituents about the benzylic carbon. Also, it is reasonable to assume that the binding interactions between the enzyme and the products and substrates involve the same nonreacting groups. If this is the case, the absolute stereochemistry of **5** at the benzylic carbon reflects the spatial arrangement of the enzyme binding sites, which are complimentary to the substituents on the benzylic carbon of the substrate. The *R* enantiomer would have the same spatial arrangement of substituents on the benzylic carbon as **5** if it were oriented for a direct displacement of the leaving group by glutathione. Under these conditions, it would be predicted that (*R*)-**3** would have greater affinity than (*S*)-**3** for the

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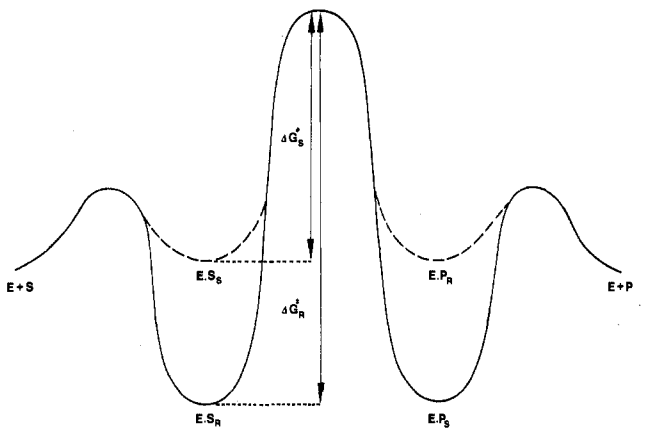
(17) Henry, D.; Block, J. H.; Anderson, J. L.; Carlson, G. R. *J. Med. Chem.* **1976**, *19*, 619.

Scheme II. Schematic Representation of the Proposed Mechanisms for the Origin of the Stereoselective Conjugation of Phenethyl Halides by Glutathione S-Transferases



enzyme. The higher affinity of (*R*)-**3** favoring the formation of a more stable enzyme–substrate complex, would result in its less efficient conversion to product. This aspect of enzymatic catalysis has been elegantly discussed by Jencks and others,[18,19] who have examined the hypothesis that enzymes derive much of their catalytic activity from their ability to express the binding energy of a specific substrate in the transition state rather than in the enzyme–substrate complex. thus, enantiomer (*R*)-**3** was converted to a product at a slower rate because a larger proportion of its intrinsic binding energy was utilized in the formation of the enzyme–substrate complex. The two proposed mechanistic origins of the stereoselectivity are not mutually exclusive and, in fact, may both be operative. These two possibilities are summarized in Scheme II.

Experimental Section

Melting points were determined in open capillary tubes on a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. IR spectra were obtained on a Perkin-Elmer 281 spectrophotometer. NMR spectra were obtained with a Varian A60D, T60D, or DFT-20 (FT-80) spectrometer. The spectra were recorded either in $CDCl_3$ containing tetramethylsilane as the internal standard or in D_2O with either sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) as the internal standard or sodium 3-(trimethylsilyl)propanesulfonate (TSP) as the external standard. All NMR spectra were recorded at ambient temperature; s refers to singlet, d to doublet, t to triplet, q to quartet, and m to multiplet. High-performance liquid chromatography (HPLC) was performed with a Waters Associates Model U6K injector, two Model M-6000 pumps, and a Beckman analytical UV detector. The column was a Waters Associates μ-Bondapak C-18, 0.46 × 25 cm. Optical rotation measurements were performed on a Perkin-Elmer Model 141 polarimeter. All UV–visible spectrophotometric determinations were performed on a Beckman DB-GT spectrophotometer.

S-Benzylglutathione was prepared from benzyl chloride by the procedure described for the preparation of *S*-(1-phenylethyl)-glutathione. *S*-Methylglutathione was obtained from Calbiochem, and reduced glutathione was purchased from Sigma Chemical Co. All chemicals and solvents were reagent grade unless otherwise stated.

***S*-[(*S*)-1-Phenylethyl]glutathione (5).** Glutathione (10 mmol, 3.07 g) was dissolved in 1.2 M KOH (25 mL, 30 mmol) under a nitrogen atmosphere. Ethanol (25 mL) was added, followed by (*RS*)-1-phenylethyl chloride (**3**; 1.4 g, 10 mmol) and a catalytic amount of 18-crown-6 (264 mg, 1 mmol). The reaction was stirred under nitrogen atmosphere at room temperature for 3 h. The mixture was adjusted to pH 3.2 with concentrated HCl, concentrated to half its volume, and extracted with ether (2 × 30 mL). Solids precipitated during extraction and concentration and were isolated by filtration. The resulting aqueous layer was concentrated to dryness in vacuo. The residue and solids were combined and recrystallized from hot water. Product enriched in **5** crystallized. The mother liquid was used for the separation of **4**. The precipitated crystals were recrystallized several times from hot water to obtain analytical samples of **5**: melting points were sharp but variable from preparation to preparation, range 204–210 dec; ^1H NMR (D_2O/Na_2CO_3/TSP) δ 7.4 (m, 5 H, C_6H_5), 4.4 (m, 1 H, SCH_2CH), 4.0 (m, 1 H, $C_6H_5CHCH_3$), 3.7 (s, 2 H, $NHCH_2COOH$), 3.6 (m, 1 H, $HOOCHNH_2$), 2.8 (m, 2 H, SCH_2CH), 2.3 (m, 2 H, CH_2CH_2CH), 2.0 (m, 2 H, CH_2CH_2CH), 1.5 (d, J = 7.0 Hz, 3 H, $C_6H_5CHCH_3$); ^{13}C NMR (D_2O, Me_3Si) δ 178.8 and 178.7 (COOH), 174.4 and 164.6 (2 CONH), 131.5, 130.2, and 129.9 (aromatic carbons), 59.0 and 57.7 (2 HNCHCOOH), 55.6 (benzylic carbon), 46.4 ($HNCH_2COOH$), 46.0 (SCH_2CH), 34.8 ($COCH_2CH_2$), 31.8 ($COCH_2CH_2CH$), 24.0 (CH_3CH). Anal. ($C_{18}H_{25}N_3O_6S$) C, H, S. The diastereomeric composition of the product was 95% of **5** and 5% of **4** as determined by HPLC.

***S*-[(*R*)-1-Phenylethyl]glutathione (4).** The mother liquid obtained after the precipitation of **5** was concentrated to provide a product enriched in **4**. Fractional crystallization from hot water provided analytical samples of **4**: mp 201.5–207.5 °C dec; ^1H NMR (D_2O/Na_2CO_3/TSP) δ 7.4 (m, 5 H, C_6H_5), 4.25 (m, 2 H, SCH_2CH, $C_6H_5CHCH_3$), 3.7 (s, 2 H, $NHCH_2COOH$), 3.3 (m, 1 H, $HOOCCHNH_2$), 2.9 (m, 2 H, SCH_2CH), 2.3 (m, 2 H, CH_2CH_2CH), 1.9 (m, 2 H, CH_2CH_2CH), 1.6 (d, J = 7.2 Hz, 3 H, $C_6H_5CHCH_3$). Anal. ($C_{18}H_{25}N_3O_6S$) C, H, S. The diastereomeric composition of the product was 70% **4** and 30% **5** as determined by HPLC.

***S*)-(−)-(1-Chloroethyl)benzene (3).** (*S*)-(+)-2-Hydroxy-2-phenylethanoic acid [1; 25 g, 164 mmol, $[\alpha]^{25}_D$ +160° (*c* 1.00, MeOH)] was dissolved in freshly distilled, dry THF (30 mL). The solution was stirred at 0 °C under nitrogen atmosphere. Borane–THF complex (290 mL, 1 M) was added slowly over a 30-min period, and the solution was stirred for 5 h at room temperature. The reaction was cooled at 0 °C and terminated by the slow addition of water (70 mL). Sodium carbonate (13 g) was added to the mixture, and the insoluble salts were removed by filtration. The organic layer was separated and washed with brine solution (2 × 250 mL). The solvent was removed in vacuo to provide a light green oil. The oil was applied to a silica gel column (175 g, 4 × 55 cm) that had been slurry packed with petroleum ether (bp 60–70 °C). Fractions of 50 mL were collected, and elution with a stepwise gradient of diethyl ether–petroleum ether (30:70, 450 mL), diethyl ether–petroleum ether (50:50, 350 mL), and diethyl ether (950 mL) was performed. Fractions 24–35, which contained the desired compound as determined by TLC (silica gel, ether), were pooled, and the solvent was removed in vacuo. Recrystallization from ether–petroleum ether provided (*S*)-(+)-1-phenyl-1,2-ethandiol as colorless needles (18.2 g, 80%): mp 62–64 °C; $[\alpha]^{23}_D$ +45.6° (*c* 1.02, MeOH) [lit.[20] mp 59–62 °C; $[\alpha]^{24}_D$ +43.2 (*c* 1.02, MeOH)]. Anal. ($C_8H_{10}O_2$) C, H, O.

A mixture of (*S*)-(+)-1-phenyl-1,2-ethandiol (18 g, 122 mmol) and dry pyridine (50 mL) was stirred at −10 to −15 °C in a methanol–ice bath. *p*-Toluenesulfonyl chloride (24.7 g, 130 mmol) was added, and the mixture was stirred at −10 °C for 4 h. The reaction mixture was diluted with ether (50 mL) and transferred into a separatory funnel. The mixture was washed with successive portions of ice-cold 10% HCl until the washes remained acidic. The ether layer was separated, shaken with an equal volume of brine solution, and dried over anhydrous sodium sulfate. The solvent was removed in vacuo to provide (*S*)-(+)-1-phenyl-1,2-ethandiol 2-tosylate (28 g, 79%), which was used without further purification.

A solution of (*S*)-(+)-1-phenyl-1,2-ethandiol 2-tosylate (28 g, 96 mmol) in dry THF (80 mL) was added dropwise to a stirred slurry of lithium aluminum hydride (10 g, 269 mmol) in dry THF (100 mL) under a nitrogen atmosphere at 0 °C. The resulting mixture was allowed to warm to room temperature and stirred

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for 3 h. Water (10 mL) was added dropwise, followed by the addition of 0.5 M NaOH (10 mL) and water (40 mL). The mixture was filtered, and the organic layer was separated, washed with brine solution, and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo to yield an amber liquid. The liquid was distilled in vacuo to give the colorless (*R*)-(+)-1-phenylethanol (**2**; 8.01 g, 68%): bp 53–54 °C (1 mmHg); $[\alpha]^{25}_D$ +45.0° (c 3.04, MeOH) [lit.[21] bp 80 °C (5 mmHg); $[\alpha]^{25}_D$ +45.9° (*c* 3.32, MeOH)]. Anal. ($C_8H_{10}O$) C, H, O.

A solution of (*R*)-(+)-**2** (1.5 g, 12.3 mmol) in dry pyridine (2.0 g, 8.5 mmol) was stirred at 0 °C. Phosphorus oxychloride (1.3 g, 8.5 mmol) in anhydrous ether (15 mL) was added dropwise over a 30-min period. After the addition was completed, the mixture was stirred at room temperature for 30 min. The mixture was cooled to 0 °C, and ice–water (20 mL) was slowly added. The ether layer was separated and washed with equal volumes of ice–water, 85% phosphoric acid, cold, saturated, aqueous sodium bicarbonate, and ice–water (×2). The ether layer was dried over anhydrous sodium sulfate, and the solvent was removed in vacuo. The resulting liquid was distilled at room temperature (0.02 mmHg) to yield **3** (0.56 g, 35%): $[\alpha]^{23}_D$ −100.6° (neat); 79.8% enantiomeric purity. Anal. (C_8H_9Cl) C, H, Cl.

(*R*)-(+)-(1-Chloroethyl)benzene (3). This compound was prepared from (*R*)-(+)-2-hydroxyphenylethanoic acid (**1**) by the same procedure used for the preparation of the *S*(−) enantiomer: $[\alpha]^{23}_D$ +99.6° (neat) [lit.[21] maximum theoretical rotation $[\alpha]^{23}_D$ +126° (neat)]; 79.0% enantiomeric purity. Anal. (C_8H_9Cl) H; C: calcd, 68.34; found, 69.02; Cl: calcd, 25.21; found, 23.96.

Preparation of Enzymes. Partially purified glutathione *S*-transferase isozymes were obtained from rat liver by a modification of the method of Habig et al.[14] Twenty male Sprague–Dawley rats (175–200 g) were fasted overnight. The rats were killed by cervical dislocation and their livers were removed, blotted dry, and weighed. The livers (130 g) were minced, placed in ice-cold distilled water (400 mL), and homogenized using a Polytron homogenizer (Brinkman Instruments). All subsequent steps were performed at 4 °C. The homogenate was centrifuged (10000*g*) (Sorvall RC-5B centrifuge DuPont Instruments) for 1 h. The supernatant (300 mL) was applied to a DEAE-cellulose column (Whatman DE-52) (5 × 16 cm) that had been previously equilibrated with 10 mM Tris–chloride buffer, pH 8.0 (buffer A). The column was rinsed with buffer A, and fractions (11 mL) were collected. The fractions were assayed for transferase activity (as described below), and fractions containing activity (fractions 13–85) were pooled. Ammonium sulfate (535 g/L) was added to the combined fractions, and the mixture was centrifuged (10000*g*) for 30 min. The precipitate was dissolved in 15 mM potassium phosphate buffer, pH 6.7 (buffer B) (150 mL) and dialyzed against buffer B (2 L × 4) for 24 h. The dialysate (200 mL) was centrifuged (105000*g*) (Beckman L2-65B ultracentrifuge) for 1 h. The supernatant was divided into two equal portions, and each was treated in the following manner. The supernatant was applied to a CM-cellulose column (Whatman CM-52) (1.5 × 30 cm) that had previously equilibrated with buffer B. Fractions of 6 mL were collected. The column was rinsed with 150 mL of bufffer B, followed by a linear gradient (1000 mL) consisting of 0 to 75 mM potassium chloride in buffer B. The fractions were monitored for transferase activity by the spectrophotometric enzyme assay.

Spectrophotometric Enzyme Assay. We determined glutathione *S*-transferase activity by measuring the initial rate of enzymatic conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione according to the procedure of Habig et al.[14] We determined the initial rate of conjugation (at 25 °C and pH 6.5) spectrophotometrically by monitoring the change in absorbance at 340 nm with time (Beckman DB-GT spectrophotometer). The activity was corrected for the nonenzymatic reaction and expressed as micromoles per minute per milligram of protein. Protein was measured by a dye-binding assay (Bio-Rad protein assay kit) with bovine serum albumin as the standard.

Determination of Glutathione in Enzyme Preparations. The glutathione concentration in the enzyme preparations was determined by the spectrophotometric procedure described by Ellman.[22] Aliquots of the enzyme preparations were diluted to 2 mL with distilled water. Potassium phosphate buffer (0.1 M, 1 mL), pH 8.0, was added, followed by 20 μL of a solution of 5.5′-dithiobis(2-nitrobenzoic acid) (10 mM in 0.1 M potassium phosphate buffer, pH 7.0). The absorbance at 412 nm was measured with a blank containing no enzyme preparation. A standard curve, generated with authentic glutathione solutions, was used to calculate the concentration of glutathione in the enzyme preparations.

HPLC Analysis of Glutathione Conjugates. The determination of the glutathione conjugates **4** and **5** in partially purified extracts from incubation mixtures was performed on a μ-Bondapak C-18 column (Waters Associates M-6000 or Beckman Model 110A pump), and a UV detector set at 254 mm was used. The amounts of the conjugates in the original incubation mixtures were determined by a standard recovery curve.[12] Known amounts of an authentic sample of *S*-(1-phenylethyl)glutathione, containing equal amounts of **4** and **5**, were substituted for the substrate **3** in the incubation mixtures. The mixtures were incubated, purified, and analyzed by HPLC. The resulting chromatographic peak heights obtained from the authentic sample were used to construct the recovery curve.

Enzyme Incubations. Enzyme incubations (10 mL total volume) were carried out at 25 °C in 0.15 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. The glutathione concentration was 5 mM in the standard experiments and 60, 53, 35, 25, and 10 μM in the low glutathione experiments. The phenethyl chloride concentration was 4 mM and was added as a solution in absolute ethanol. The final ethanol concentration in the incubation mixture was adjusted to 4% (v/v). The incubations proceeded for 5 min, and the reactions were terminated by the addition of 20% trichloroacetic acid solution (2 mL). The precipitated protein was removed by centrifugation, and the glutathione conjugates were extracted from the supernatant as previously described.[12]

Inhibitory Enzyme Kinetics. The inhibition by the glutathione conjugates, **4**, **5**, *S*-methylglutathione, and *S*-benzylglutathione, of the transferase A catalyzed conjugation of CDNB with glutathione was determined by monitoring the initial rates of product formation at 340 mn in the presence of 20, 40, 60, 100, 150, and 200 μM concentrations of the inhibitors. Four fixed concentrations of glutathione and CDNB were used (glutathione and CDNB concentrations were, respectively, 10 and 0.7, 10 and 0.2, 0.5 and 0.7, and 0.5 and 0.2 mM in 2.5% ethanol and 0.15 M potassium phosphate buffer, pH 6.5). All of the inhibitory kinetics were analyzed by the method of Dixon.[23]

Acknowledgment. J.B.M. was supported by Training Grant GM-07480 from the National Institutes of Health and BRS Grant 5S01-RR05634. The authers thank William B. Jakoby, Ph.D. (NIH), for providing samples of the antisera for glutathione *S*-transferase A.

Registry No. (*S*)-**1**, 17199-29-0; (*R*)-**1**, 611-71-2; (*R*)-**2**, 1517-69-7; (*S*)-**3**, 3756-41-0; (*R*)-**3**, 1459-15-0; (*RS*)-**3**, 38661-82-4; **4**, 75878-94-3; **5**, 75813-49-9; *S*-benzylglutathione, 6803-17-4; benzyl chloride, 100-44-7; *S*-methylglutathione, 2922-56-7; glutathione, 70-18-8; (*S*)-(+)-1-phenyl-1,2-ethandiol, 25779-13-9; (*S*)-(+)-1-phenyl-1,2-ethandiol 2-tosylate, 40435-14-1; glutathione *S*-transferase, 50812-37-8; CDNB, 97-00-7.

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