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GLUTATHIONE CONJUGATION OF STYRENE 7,8-OXIDE ENANTIOMERS BY MAJOR GLUTATHIONE TRANSFERASE ISOENZYMES ISOLATED FROM RAT LIVERS

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Abstract—Male Sprague–Dawley rat liver cytosol mediated regioselective conjugation of styrene 7,8-oxide (STO) enantiomers with glutathione in completely *trans*-ring-opening manner to afford (1*S*)-*S*-(1-phenyl-2-hydroxyethyl)glutathione and (2*R*)-*S*-(2-phenyl-2-hydroxyethyl)glutathione in the ratio 22:1 for (*R*)-STO and also to afford (1*R*)-*S*-(1-phenyl-2-hydroxyethyl)glutathione and (2*S*)-*S*-(2-phenyl-2-hydroxyethyl)glutathione in the ratio 12:1 for (*S*)-STO. In the above cytosolic reactions, (*R*)-STO was conjugated 1.8 times faster than (*S*)-STO, while the (*R*)- to (*S*)-ratio in rate of the conjugation was 2.7 when racemic STO was used as a substrate. A kinetic study, carried out by using six major glutathione transferase (GST) isoenzymes isolated from the cytosol, indicated that GSTs 3-3, 3-4 and 4-4 (class *mu* enzymes) had much higher K_{cat}/K_m values towards both STO enantiomers than the other three major isoenzymes, GSTs 1-1, 1-2 and 2-2 (class *alpha* enzymes). All the class *mu* enzymes mediated preferential glutathione conjugation of (*R*)-STO to (*S*)-STO. On the contrary, the class *alpha* enzymes catalysed the conjugation of (*S*)-STO preferentially to (*R*)-STO. The kinetic study strongly suggested that GSTs determining the higher enantioselectivity towards (*R*)-STO in the rat liver cytosol were the class *mu* enzymes, especially GST 3-3, which had the highest K_{cat}/K_m value towards (*R*)-STO as well as the highest (*R*) to (*S*) ratio in the enantioselectivity among the six isoenzymes examined. GST 7-7, isolated as a major enzyme from the liver cytosol of the animals bearing hepatic hyperplastic nodules which were induced by chemical carcinogens, catalysed preferential GSH conjugation of (*S*)-STO to (*R*)-STO.

Styrene 7,8-oxide (STO or 2-phenyloxiran) that induces tumors in the mouse skin on painting [1] and at the forestomach of rats on administration with drinking water [2] is a reactive key intermediate in the metabolism of the plastic monomer styrene to two types of major metabolites in the rat liver; one is styrene 7,8-glycol (STG or phenylethane-1,2-diol), a hydrolysis product by microsomal epoxide hydrolase (EH) [3–8], and the other glutathione (GSH) conjugates by cytosolic GSH *S*-transferases (GSTs) in the presence of GSH [8–10]. Rats given styrene or racemic STO i.p. excrete enantiomeric mixtures of STG [11], mandelic acid [11], phenylglyoxalic acid [11] and hippuric acid [11, 12] through the hydrolysis route and four diastereomeric phenylhydroxyethyl mercapturic acids through the GSH conjugation pathway [11–14].

Epoxidation by microsomal cytochromes P-450 is a rate-limiting step in the hepatic metabolism of styrene in the rat, and the resulting STO which has only a little difference in *R* to *S* ratio ($R < S$) [7] is immediately scavenged by microsomal EH ($R \ll S$) [7] and cytosolic GSTs ($R \gg S$) with greater enantioselectivity [8, 15]. (*R*)-STO has higher mutagenicity towards *Salmonella typhimurium* TA 100 than did (*S*)-STO [16]. In the presence of GSH, GSH conjugation of racemic STO occurs much faster than its enzymic hydrolysis in the rat liver post-mitochondrial fraction [17].

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STO would be an only instance of yielding regioisomeric GSH conjugates of olefin oxides with benzylic and non-benzylic oxiran carbons [10, 15]. The epoxides, 2-alkyl-3-aryl-oxirans, such as *trans*-8-methylstyrene oxide [15, 18], 1,2-dihydronaphthalene 3,4-oxide [15, 18], 1,2-dihydrophenanthrene 3,4-oxide [18], and 9,10-dihydrobenzo[*a*]pyrene 7,8-oxide [19], are all conjugated specifically at their benzylic carbons with GSH by rat liver cytosolic GSTs. However, the 2-alkyl-2-aryl-oxiran, 7-methylstyrene oxide, is regiospecifically conjugated with GSH at the non-benzylic oxiran carbon in the rat liver cytosol [15].

Racemic STO was demonstrated by Bend and his coworkers [15] to yield four diastereomeric GSH conjugates, *S*-(phenylhydroxyethyl)glutathiones, two of which were major products resulting from conjugations at the benzylic carbon (C_7) of the racemic epoxide, and the other two minors at the non-benzylic carbon (C_8). No direct information, however, has been obtained with (*R*)- and (*S*)-STOs of whether the enantiomeric epoxides are enzymically conjugated in *trans*-ring-opening manner, like epoxides of cyclic olefins and arenes, or not.

Very recently, we have demonstrated that major dimeric GST isoenzymes, consisting of subunit proteins 1, 2, 3 and 4 which correspond to more than 98% of the total GST proteins existing in the rat liver cytosol, show a marked difference in catalytic activity towards racemic STO; GSTs 3-3, 3-4, and 4-4 (class *mu* enzymes) played much more important

parts in conjugating the racemate than GSTs 1-1, 1-2, and 2-2 (class *alpha* enzymes) [20]. In the present paper, we wish to report that the enantiomers (*R*)- and (*S*)-STOs, are regioselectively conjugated with GSH at their benzylic carbons in completely *trans*-ring-opening manner with enantioselectivity of (*R*)- > (*S*)-STOs in the rat liver cytosol and also that, of the major GSTs, the class *mu* enzymes play important roles in determining the higher enantioselectivity towards (*R*)-STO in the rat liver cytosol.

MATERIALS AND METHODS

Materials. Racemic STO and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). GSH, free from its oxidized form, was donated by Yamanouchi Pharmaceutical Co. Ltd (Tokyo, Japan). Racemic STO was distilled under reduced pressure before use. Racemic [7-^{3}H]STO (209 mCi/mmol) was purchased from Radiochemical Centre (Amersham, U.K.), diluted with redistilled STO, and purified by high pressure liquid chromatography (HPLC) on a silica gel column as previously reported [20] to obtain a specimen with radiochemical purity higher than 99%, based on the radioactivity to absorbancy (254 nm) ratio.

(*R*)- and (*S*)-STOs with enantiopurity higher than 99% were synthesized from (*R*)- and (*S*)-mandelic acids (Aldrich Chemical Co. Inc, Milwaukee, WI) with enantiomeric purity higher than 99% via (*R*)- and (*S*)-1-phenyl-2-*p*-toluenesulphoxyethanols as previously reported [21]. (1*R*)- and (1*S*)-1-phenyl-2-hydroxyethylmercapturic acid methyl esters (methyl esters of (1*R*)- and (1*S*)-*N*-acetyl-*S*-(1-phenyl-2-hydroxyethyl)-L-cysteines) and (2*R*)- and (2*S*)-2-phenyl-2-hydroxyethylmercapturic acid methyl esters (methyl esters of (2*R*)- and (2*S*)-*N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)-L-cysteines) were synthesized and purified as diastereomerically pure specimens by the method of Delbressine *et al.* [14].

(R)- *and* (S)-[7-^{3}H]*STOs.* (*R*)- and (*S*)-[7-^{3}H]STOs with enantiomeric and radiochemical purities both higher than 99% were prepared as follows: to 1 N sulphuric acid (3 ml) was added a solution of the aforementioned, chromatographically purified racemic [7-^{3}H]STO (2 mCi, 9.57 μmol) in acetone (0.5 ml), and the mixture was kept at 40° for 90 min and then washed with *n*-hexane (2 ml). The aqueous phase was saturated with sodium chloride and extracted twice with ethyl acetate. The combined ethyl acetate extract was washed with a 5% aqueous sodium bicarbonate solution (0.5 ml), which was presaturated with sodium chloride, and then dried over anhydrous magnesium sulfate. After the evaporation of the solvent, the radioactive residue, containing racemic [1-^{3}H]1-phenylethane-1,2-diol (1.4 mCi), was dissolved in anhydrous pyridine (100 μl). To the radioactive solution was added a solution of *p*-toluenesulphonyl chloride (21 μmol) in anhydrous pyridine (50 μl), and then the mixture was kept at room temperature for 5 hr. The reaction mixture was diluted with water (0.5 ml) and extracted twice with *n*-hexane (1.0 ml).

The combined extract was shaken with a 5% aqueous cupric sulphate solution (1 ml), dried over anhydrous sodium carbonate and condensed to dryness *in vacuo*. Racemic [1-^{3}H]1-phenyl-2-*p*-toluenesulphonylethanol in the residue was purified by HPLC on a silica column (LiChrosorb Si-60, 10 μm in particle size, 4 × 250 mm) in tetrahydrofuran-*n*-hexane (5:95, v/v, 1.0 ml/min) by monitoring the chromatogram at 254 nm; the retention time was 16 min. The chromatographically purified, racemic radioactive monotoluenesulphonate (820 μCi) was subjected to HPLC on a chiral column (CHIRALCEL OB, Daicel Chemical Industries, Ltd, Tokyo; 10 μm in particle size, 4.6 × 250 mm) in isopropanol-*n*-hexane (40:60, v/v, 1 ml/min). The (*R*)- and (*S*)-enantiomers of the radioactive monotoluenesulphonate were eluted at 15 and 29 min, respectively, under the HPLC conditions.

Chromatographically separated (*R*)- and (*S*)-[1-^{3}H]1-phenyl-2-*p*-toluenesulphoxyethanols (100 μCi, 0.48 μmol) were quantitatively converted at 0° within 1 hr into (*R*)- and (*S*)-[7-^{3}H]STOs, respectively, in methanol (0.5 ml) containing potassium hydroxide (10 mg). The reaction mixture was diluted with water (1.0 ml) and extracted twice with *n*-hexane (1.0 ml each). The combined organic layer was dried over anhydrous sodium carbonate and condensed in a distilling flask equipped with a fractional condenser under a gently refluxing condition. The residue of each [7-^{3}H]STO enantiomer had a radioactivity of about 88 μCi and showed an only UV-absorbing peak at 254 nm due to STO in the HPL-chromatogram obtained on the CHIRALCEL OB column in isopropanol-*n*-hexane (7:93, v/v, 1.0 ml/min); the retention times were 10.6 min for both (*R*)- and (*S*)-STOs. (*R*)- and (*S*)-[7-^{3}H]STOs, thus obtained, had the same specific activity (208.3 μCi/mol) and radiochemical purity (>99%), based on the radioactivity to absorbance (254 nm) ratio before and after HPLC of the enantiomers.

Liver cytosol and GSTs. Liver cytosol was obtained from male Sprague–Dawley (SD) rats, weighing 150–180 g, as previously reported, dialysed at 2° for 24 hr against 0.1 M KH_2PO_4–K_2HPO_4 buffer, pH 7.4 (2500 vol.), and adjusted with the same buffer to a protein concentration of 21 mg/ml.

GSTs 1-1, 1-2, 2-2, 3-3, 3-4 and 4-4 were isolated from the normal rat liver cytosol by the method of Mannervik and Jensson [22], and GST 7-7 was from the liver cytosol of the animals bearing hepatic hyperplastic nodules induced by hepatocarcinogens, by the method of Satoh *et al.* [23]. Homogeneity of the GST isoenzymes was confirmed by two-dimensional gel electrophoresis (isoelectric focusing/sodium dodecyl sulphate electrophoresis) [23, 24] as well as by the double immuno-diffusion test as previously reported [22, 23]. The GST isoenzymes used had the following specific activities towards CDNB (μmol/mg protein/min) determined as previously reported [23, 25]: 1-1 (33.0), 1-2 (26.0), 2-2 (17.2), 3-3 (28.3), 3-4 (32.9), 4-4 (19.6) and 7-7 (15.2)

Isolation and derivatization of GSH conjugates to mercapturic acid methyl esters. After incubations of (*R*)-, (*S*)- and racemic STOs with the dialysed cytosol from rat liver in the presence of GSH, the incubation mixtures (15 ml, see the legend of Fig. 1 with the

incubation conditions and concentrations of the ingredients), were immediately chilled and shaken with ethyl acetate (5 ml) to remove the unreacted substrates and most of phenylethane-1,2-diols. The separated residual aqueous phase was washed with ether to remove ethyl acetate, evacuated by aspiration to remove ether, and poured onto an Amberlite XAD-2 resin column (1 × 25 cm). The resin column was rinsed with water and 50% (v/v) methanol (3 bed vol. each) and eluted with methanol (3 bed vol.). The methanolic eluate was condensed to dryness *in vacuo* and subjected to HPLC under the same conditions as previously reported by Hernandez *et al.* [26] to separate the diastereomers of the GSH conjugates. The diastereomeric GSH conjugates separated were rechromatographed under the same HPLC conditions for further purification and subsequent derivatization into the corresponding mercapturic acid methyl esters.

Mercapturic acid methyl esters were obtained from the chromatographically separated GSH conjugates as follows: to a solution (0.5 ml) of the GSH conjugate (1–15 μmol) in 0.1 M KH_2PO_4–K_2HPO_4 buffer, pH 7.4, a dialysed postmitochondrial fraction (1.5 ml) of a kidney homogenate from the SD rats was added and incubated at 37° for 18 hr. The rat kidney subcellular fraction was obtained by centrifugation of a three volume homogenate of the rat kidneys in isotonic KCl at 9000 *g* for 30 min and then by dialysis of the supernatant fraction against 0.1 M KH_2PO_4–K_2HPO_4, pH 7.4 (100 vol.). The incubation mixtures, containing cysteine conjugates formed from the GSH conjugates, were poured onto Amberlite XAD-2 columns (1 × 25 cm). The resin column was rinsed successively with water and 50% (v/v) aqueous methanol (3 bed vol. each) and eluted with methanol (3 bed vol.). The methanolic eluate was condensed to dryness, and the residue was dissolved in 1 N sodium hydroxide (1 ml). To the aqueous alkaline solution was added acetic anhydride (0.2 ml), and the mixture was kept at room temperature for 1 hr with stirring, acidified with hydrochloric acid to pH 2, saturated with sodium chloride, and extracted three times with ethyl acetate (2 ml each). The combined organic extract, containing a mercapturic acid, was washed with sodium chloride-saturated water and condensed to dryness *in vacuo*. The residue obtained was dissolved in cold ether containing an excess amount of diazomethane. After 30 min at 2°, the organic solvent was evaporated to dryness, and the residue obtained was dissolved in chloroform for subsequent purification of the mercapturic acid methyl ester by HPLC (see the legend of Table 1 with the HPLC conditions).

Kinetic parameters. K_m and K_{cat} values for GSH conjugations of the STO enantiomers by GST isoenzymes were estimated from double reciprocal plot of the substrate concentrations, ranging from 0.1 to 2.0 mM, versus the apparent reaction rates obtained in the zero-order kinetics region as follows: mixtures consisting of GSH (4 μmol), normal rat liver cytosol (0.125 mg protein) or GSTs (21–50 μg protein), and 0.1 M KH_2PO_4–K_2HPO_4, pH 7.4 (1.0 ml), were preincubated at 37° for 5 min in air-tight 10 ml test tubes. The reaction was started by the addition of a solution of (*R*)- or (*S*)-[7-^{3}H]STO (0.1 μCi) in acetone (50 μl). Incubation was carried out for 10 min. Unlabelled (*R*)- or (*S*)-STO used as an inhibitor was dissolved in the acetone containing the radioactive substrate. After the incubation, the mixture was immediately chilled on an ice bath and rapidly shaken with ethyl acetate (2 ml) which was presaturated with the potassium phosphate buffer. The residual aqueous phase was saturated with sodium chloride and extracted twice with ethyl acetate (2 ml each) which was presaturated with potassium phosphate buffer containing a saturating amount of sodium chloride. The washed aqueous phase which was free from radioactive phenylethane-1,2-diol was diluted with an appropriate volume of water and then with a dioxan scintillator to count the radioactivity of the GSH conjugates of [7-^{3}H]STO with an Aloka model 1000 liquid scintillation counter. Blank runs were carried out by using boiled rat liver cytosol or GSTs under the same incubation conditions.

Proteins of rat liver cytosol and GSTs were determined by the method of Lowry *et al.* [27].

Chromatography and spectroscopy. HPLC was carried out with a JEOL model 1000 liquid chromatograph equipped with a UVIDEC-100-VI UV monitor, thin-layer chromatography (TLC) on Wakogel B5F plates in *n*-butanol–acetic acid–water (4:1:1, v/v/v), and gas–liquid chromatography–mass spectroscopy (GLC–MS) with a Shimadzu model QP-1000 gas chromatograph–mass spectrometer. UV absorption spectra were recorded in water or ethanol with a Hitachi model 557 spectrometer.

RESULTS

Regioselective GSH conjugation of STO enantiomers at their benzylic carbons by SD rat liver cytosol

HPLC of GSH conjugates, formed from enantiomerically pure (*R*)- and (*S*)-STOs by male SD rat liver cytosol in the presence of GSH and then quantitatively isolated from the incubation mixtures after removal of the substrates and the minor hydrolysis products STG, indicated that cytosolic GSH conjugation of each enantiomeric epoxide yielded only two products of four possible diastereomers with a large difference in ratio; their retention times were 33.2 and 46.0 min for the major (conjugate I) and minor (conjugate II) products of (*R*)-STO, respectively, and 38.8 and 42.9 min for the major (conjugate III) and minor (conjugate IV) products of (*S*)-STO, respectively (Fig. 1). From racemic STO, however, the four chromatographically separable conjugates, two majors I and III and two minors II and IV, were formed under the same incubation conditions. Nonenzymic GSH conjugation of these STOs also occurred at a very small rate, less than 1% of the enzymic rate, at pH 7.4 used for the cytosolic conjugation reactions.

The four GSH conjugates, separated and purified to homogeneity by HPLC, showed the same R_f value of 0.31 on a silica plate, visualized by ninhydrin and with a UV lamp. Each GSH conjugate was almost quantitatively derivatized into the corresponding *S*-cysteine conjugate by the treatment with a rat kidney 9000 *g* supernatant fraction (S9), containing potent activities of γ-glutamyl transpeptidase and cysteinyl glycinase. *S*-Cysteine conjugates obtained were then

Table 1. Identification of GSH conjugates formed from STO enantiomers by derivatization into mercapturic acid methyl esters

GSH conjugate*	Mercapturic acid methyl ester formed: Structure	HPLC† (min)	GLC‡ (min)	GLC–MS‡ m/z (relative intensity, %)
From (*R*)-STO				
Conjugate I (major)	(1*S*)-1-Phenyl-2-hydroxyethylmercapturic acid methyl ester (PHEM I)	28.2	8.3	279(M^+-H_2O, 28), 267(36.1), 236(36.1), 161(25), 144(22), 143(33), 123(36), 122(25), 121(81), 104(100), 103(33), 91(75), 89(33)
Conjugate II (minor)	(2*R*)-2-Phenyl-2-hydroxyethylmercapturic acid methyl ester (PHEM II)	20.3	7.6	297(M^+, 17), 279(31), 236(36), 235(28), 191(72), 190(22), 132(100), 107(72), 100(28), 89(31)
From (*S*)-STO				
Conjugate III (major)	(1*R*)-1-Phenyl-2-hydroxyethylmercapturic acid methyl ester (PHEM III)	25.1	8.3	279(M^+-H_2O, 28), 267(36), 236(39), 161(25), 144(22), 143(33), 123(36), 122(25), 121(81), 104(100), 103(33), 91(75), 89(33)
Conjugate IV (minor)	(2*R*)-2-Phenyl-2-hydroxyethylmercapturic acid methyl ester (PHEM IV)	18.3	7.6	297(M^+, 14), 279(31), 236(36), 235(28), 191(72), 190(25), 132(100), 107(72), 100(28), 89(28)

* GSH conjugates were separated and purified by HPLC as shown in Fig. 1, hydrolysed by the rat kidney 9000 *g* supernatant fraction to the corresponding cysteine conjugates which were isolated by HPLC, N-acetylated with acetic anhydride to the mercapturic acids, and O-methylated with diazomethane to the methyl esters as described in Materials and Methods. The methyl esters were identified with the corresponding authentic diastereomers prepared and purified by the method of Delbressine *et al.* [14].

† A silica column (LiChrosorb Si-60, 5 μm in particle size, 4 × 250 mm) was eluted with chloroform (2 ml/min), and the chromatograms were monitored at 254 nm.

‡ A GLC column (2% OV-17 coated on Chromosorb W, 60–80 mesh, 4 mm × 2 m) was eluted with helium (48 ml/min) at 250°. The temperatures of the injection port and ionization chamber were 270° and 250°, respectively. The ionization energy was 70 eV.

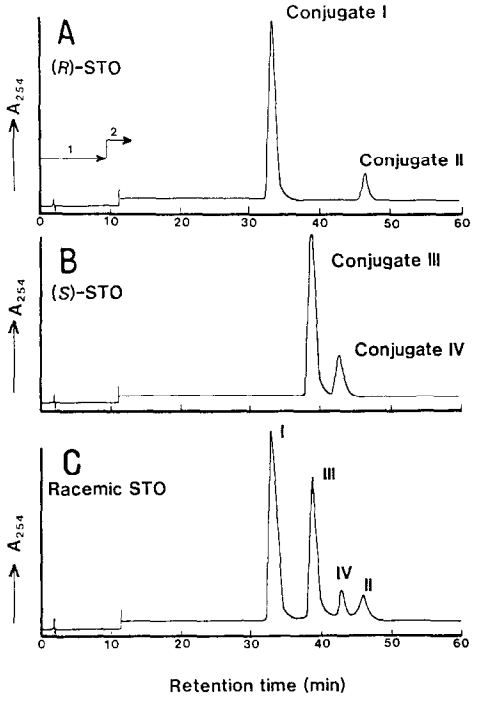

Fig. 1. Separation by HPLC of regioisomeric GSH conjugates formed from enantiomeric and racemic STOs by rat liver cytosol in the presence of GSH. The epoxides (10 μmol) dissolved in acetone (0.1 ml) were incubated at 37° for 20 min with the dialysed cytosol (0.54 mg protein) from rat liver in the presence of GSH (20 μmol) in a final volume of 5 ml 0.1 M KH_2PO_4–K_2HPO_4, pH 7.4, in air-tight flasks. A C_{18}Radial-PAK column (5 μm in particle size, 8 × 150 mm, Waters Associates, Milford, MA) was eluted at a flow rate of 2 ml/min by the step-gradient method by using solvent systems 1 (25 mM Tris–phosphate buffer, pH 7.0–25 mM sodium sulphate) and 2 (solvent system 1 containing 2.5% v/v methanol). 4-*S*-glutathionyl-3-hydroxy-1,2,3,4-tetrahydrophenanthrene used as an internal reference was eluted at 120 min. Chromatogram C was twice enlarged in absorbancy.

derivatized into phenyl-hydroxyethyl-mercapturic acids (PHEMs) by sequential treatments with acetic anhydride and diazomethane as illustrated in Fig. 2.

PHEMs I-IV, derivatized as single products from the respective GSH conjugates, were purified by HPLC on a silica column and identified by GLC–MS with the corresponding synthetic specimens (Table 1). The four PHEMs were eluted from the silica HPLC column at different retention times and showed mass spectra characteristic of their regioisomeric structures. From the assigned absolute configurations of synthetic PHEMs, the major GSH conjugates formed from the STO enantiomers were identified as (1*S*)-*S*-(1-phenyl-2-hydroxyethyl)-glutathione for conjugate I (from (*R*)-STO) and as its (1*R*)-isomer for conjugate III (from (*S*)-STO), both of which were *trans*-ring-opening-products formed by the attack of the sulfhydryl group of GSH on the benzylic oxiran carbons (C_7) of STOs with inversion of their absolute configurations (Fig. 2). Similarly, the minor GSH conjugates were identified as (2*R*)-*S*-(2-phenyl-2-hydroxyethyl)glutathione for conjugate II (from (*R*)-STO) and as its (2*S*)-isomer for conjugate IV (from (*S*)-STO), both formed by the attack of GSH on the non-benzylic oxiran carbons (C_8) of STOs in the same stereochemical manner without inversion of their C_7 configurations as mentioned above.

The ratio of conjugates I to II formed from (*R*)-STO in the cytosol, was 22:1, while that of conjugates III to IV from (*S*)-STO was 12:1 (Fig. 1). There was a considerable difference in the rate of the enzymic conjugation of the STO enantiomers: (*R*)/(*S*) = 1.8. However, the difference was much larger when racemic STO was used as a substrate: (*R*)/(*S*) = 2.7. These data of enantioselectivity in GSH conjugation were based on the ratio of the total amount of conjugates I and II to that of conjugates III and IV formed from the STO enantiomers or the (*R*)- and (*S*)-components of the racemate.

Little difference was observed in regioselectivity in the enzymic reactions between the enantiomeric components of the racemate and the corresponding STO enantiomers used independently.

Non-enzymic reactions in GSH conjugation of the STO enantiomers occurred at an exponentially higher rate at higher pH up to 10 to afford the *trans*-ring-opening products specifically. At pH 10, the non-enzymic reactions proceeded at a rate higher than the enzymic reactions with little difference in rate and regioselectivity between the STO enantiomers; the ratio of the major to minor conjugates was 10:1 from both enantiomers.

Inhibition of hepatic cytosolic GSH conjugation of STO enantiomers by their enantiomeric counterparts

Apparent V_{max} and K_m values, obtained by the double reciprocal plot method in the hepatic cytosolic GSH conjugation reactions, were 66.7 nmol/mg protein/min and 0.7 mM for (*R*)-STO and 100 nmol/mg protein/min and 2.0 mM for (*S*)-STO. The enzymic conjugation reactions of the STO enantiomers were competitively inhibited by their enantiomeric counterparts (Fig. 3). In the racemic mixtures (0.5 and 1 mM), consisting of equal half molar ratios of (*R*)- and (*S*)-STOs, either of which was labelled with tritium, formations of radioactive GSH conjugates from (*R*)- and (*S*)-[7-^{3}H]STOs were inhibited by 35 and 59% with unlabelled (*S*)- and (*R*)-STOs, respectively.

GSH conjugations of STO enantiomers by major GSTs isolated from SD rat liver cytosols

(*R*)- and (*S*)-[7-^{3}H]STOs were incubated under the aforementioned conditions with homogeneous preparations of the major GST isoenzymes, GSTs 1-1, 1-2, 2-2, 3-3, 3-4 and 4-4, isolated from the normal SD rat liver cytosol and of GST 7-7 isolated as a major GST protein from the liver cytosol of SD rats bearing hepatic hyperplastic nodules induced by hepatocarcinogens. The isoenzymes showed marked differences in catalytic activity for GSH conjugations of the STO enantiomers (Table 2). GSTs 1-1, 1-2, and 2-2 (class *alpha* enzymes) catalysed GSH conjugations of the STO enantiomers at very low rates with difference in enantioselectivity ((*S*)>(*R*) for GSTs 1-1 and 1-2 and (*S*)≈(*R*) for GST 2-2) from those mediated by the rat liver cytosol ((*R*)>(*S*) as mentioned above). Both (*R*)- and (*S*)-STOs not only had a very low affinity for these isoenzymes so far as

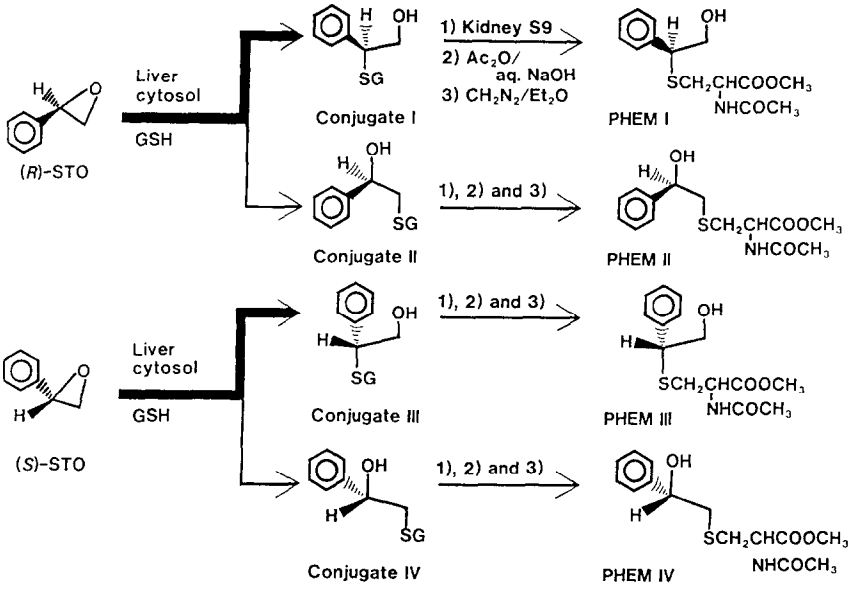

Fig. 2. Derivatization of regioisomeric GSH conjugates formed from STO enantiomers into the corresponding mercapturic acid methyl esters after separation by HPLC. S9 represents a 9000 *g* supernatant fraction of the rat kidney homogenate.

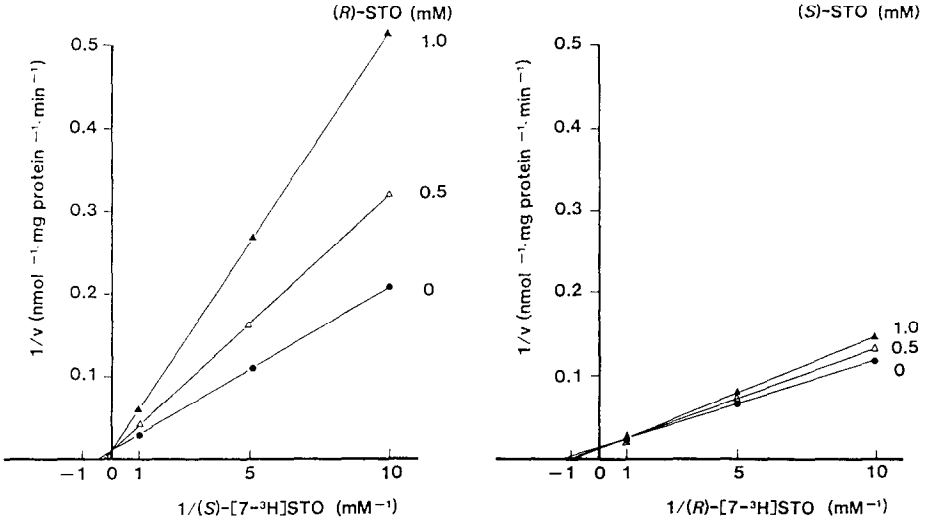

Fig. 3. Inhibition of hepatic cytosolic GSH conjugations of [7-^{3}H]STO enantiomers by their unlabelled enantiomeric counterparts. (*R*)- and (*S*)-[7-^{3}H]STOs (0.1 μCi/ml, 0–1.0 mM) were incubated with rat liver cytosol (0.125 mg protein/ml) in 0.1 M KH_2PO_4–K_2HPO_4 buffer, pH 7.4 in the absence (●) and in the presence of 0.5 mM (Δ) and 1.0 mM (▲) of the unlabelled enantiomeric counterparts. Radioactive GSH conjugates formed were determined by direct measurement of the radioactivity of the aqueous phase obtained after extractions with ethyl acetate of the incubation mixture saturated with sodium chloride. Blank control runs were carried out by using boiled cytosol under the aforementioned conditions. The data were expressed as arithmetic mean values of at least four experiments after subtraction of the radioactivities for the blank control runs from the total radioactivities for the enzymic reactions.

estimated by the K_m values, but also showed low K_{cat} values.

In contrast with the class *alpha* enzymes, GSTs 3-3, 3-4 and 4-4 (class *mu* enzymes) catalysed the GSH conjugations at high rates. The class *mu* enzymes showed much lower K_m values and much higher K_{cat} values towards the STO enantiomers (Table 2). In addition, all of the class *mu* enzymes had higher K_{cat}/K_m values towards (*R*)-STO than towards (*S*)-STO; the (*R*) to (*S*) ratios of these values were 2.9, 1.7 and 1.5 for GSTs 3-3, 3-4 and 4-4, respectively. GST 7-7, a negligibly minor GST component in the normal rat liver cytosol, had a 2.4 times higher K_{cat}/K_m value towards (*S*)-STO than towards (*R*)-STO.

DISCUSSION

The present study was carried out by using the

Table 2. Kinetic parameters for GSH conjugation of STO enantiomers by major GST isoenzymes isolated from rat liver cytosols

GST*	(*R*)-STO			(*S*)-STO		
	K_{cat}†	K_m	K_{cat}/K_m	K_{cat}†	K_m	K_{cat}/K_m
1-1 (Ya-Ya)	0.004	2.5	0.0016	0.01	3.0	0.003
1-2 (Ya-Yc)	0.008	2.2	0.0036	0.02	4.0	0.005
2-2 (Yc-Yc)	0.012	5.6	0.0015	0.02	10.0	0.002
3-3 (Yb_1-Yb_1)	0.47	0.6	0.78	0.27	1.0	0.27
3-4 (Yb_1-Yb_2)	0.35	0.5	0.71	0.29	0.7	0.41
4-4 (Yb_2-Yb_2)	0.20	0.4	0.50	0.26	0.8	0.33
7-7 (Yp-Yp)	0.11	2.1	0.05	0.24	2.0	0.12

K_{cat} is expressed as per sec, and K_m as millimolar. Incubation conditions are described in Materials and Methods.

* GSTs were isolated from normal male SD rat liver cytosol except for GST 7-7 from the liver cytosol of the animals bearing hepatic hyperplastic nodules induced by hepatocarcinogens.

† The molecular weights of GSTs are based on Ketterer *et al.* [28].

same HPLC system as proposed by Hernandez *et al.* [26] who had established the complete separation of the four diastereomers of the GSH conjugates formed from racemic STO. There existed a marked difference in enantioselectivity between hepatic cytosolic GSH conjugations of STO enantiomers used as substrates ($(R)/(S) = 1.8$) and of the enantiomeric components existing in racemic STO ($(R)/(S) = 2.7$). That would be attributable to the large difference in affinity of the STO enantiomers for the cytosolic GSTs.

The kinetic study carried out with mixtures of various molar ratios of (*R*)-[7-^{3}H]STO to (*S*)-STO and of (*S*)-[7-^{3}H]STO to (*R*)-STO indicated that cytosolic formation of the radioactive conjugates from radioactive STOs were both strongly retarded by the unlabelled counterparts. However, the radioactive conjugate formation from the mixtures of (*S*)-[7-^{3}H]STO and unlabelled (*R*)-STO was more strongly retarded than that from the other mixtures as readily assumed from the observed K_m values ((*R*)- <(*S*)-STOs) in GSH conjugation of the enantiomeric epoxides in the rat liver cytosol.

Considering the reported concentrations of the known GST proteins in the normal rat liver cytosol (GSTs 1-1 > 1-2 > 3-4 > 3-3 > 4-4 ≃ 2-2 > 4-6 [28] 8-8 [29] ≫ 5-5 [28]), the isoenzymes determining the enantioselectivity in GSH conjugations of the racemate and enantiomers of STO ((*R*) > (*S*)) could be attributed to the class *mu* enzymes because they had been demonstrated to correspond to about 40% of total GST proteins in the rat liver [28] and showed higher turn-over rates (larger K_{cat}/K_m) with higher affinity (smaller K_m) towards (*R*)-STO than towards (*S*)-STO in the present study. The class *alpha* enzymes, the most abundant GST proteins, corresponding to about 60% of the total rat liver cytosolic GSTs [28], were demonstrated in the present study as well as in our previous study carried out by using racemic [7-^{3}H]STO to play only a minor role in detoxifying the carcinogen STO, compared with the class *mu* enzymes [20].

However, it is of interest that GSTs 1-1 and 1-2 play an almost specific role in conjugating aflatoxin B_1 8,9-oxide [30] and cholesterol 5,6α-epoxide [31, 32], the latter of which is a suggested causative material for human colon cancer [33] and formed from cholesterol by lipid peroxidation of hepatic microsomal phospholipids [34]. On the contrary, the class *mu* enzymes have been demonstrated to mediate GSH conjugation of a variety of carcinogenic or mutagenic epoxides of polycyclic aromatic hydrocarbons (PAHs), such as *K*-region oxides of PAHs [35–37] and aza-PAHs [37, 38] and benzo[*a*]pyrene (B*a*P)-7,8-diol 9,10-oxides [39–41]. Of the three isoenzymes of this class, GST 4-4 catalyses highly enantioselective GSH conjugation with the (*R*)-oxiran carbons of the *K*-region oxides of PAHs (>99%) [38] and of the bay region dioloxides of B*a*P, benz[*a*]anthracene and chrysene (>95%)[40]. GST 3–4 shows less enantioselectivity towards the (*R*)-oxiran carbons of the *K*-region oxides and B*a*P-7,8-diol 9,10-oxides than GST 4-4, and there is little enantioselectivity in GSH conjugation of these enantiomeric epoxides by GST 3-3 [37, 38, 41]. Very similar enantioselectivity has recently been reported by us with enantiomeric 9,10-dihydro-B*a*P 7,8-oxides (DBPOs) which are regiospecifically conjugated at the benzylic carbon(C_7) with GSH not only in the normal SD rat liver cytosol, but also by the major GSTs isolated therefrom [42]. The twice higher enantioselectivity towards (7*R*)-DBPO than towards (7*S*)-DBPO in GSH conjugation by the liver cytosol was found to be determined mainly by GSTs 3-4 and 4-4, but not by GST 3-3 which had little selectivity towards the enantiomers [42]. The enantiomers of DBPO were conjugated at very low rates by the class *alpha* enzymes. In the present study, however, GST 3-3 showed the highest enantioselectivity towards (*R*)-STO among the class *mu* enzymes. Actually, GST 3-3 catalysed the most preferential conjugation of (*R*)-STO to (*S*)-STO among the class *mu* enzymes when racemic STO was used as a substrate (data not shown).

Of the other cytosolic GST isoenzymes in the rat liver, only GST 5-5, a very minor isoenzyme (<0.2% of the total rat liver GST proteins) [43], has been examined for GSH conjugation of racemic STO [44]. Racemic STO, however, has been well studied with purified GST preparations from human liver cytosol [45, 46]. The human liver data indicate that the conjugation of racemic STO is catalysed by its isoenzymes in order of GSTs $\mu > \pi > \alpha \sim \varepsilon$. This fact is of interest in view of the homology of the rat liver

GSTs to the human isoenzymes because it has been demonstrated that human GSTs μ, π and $\alpha\sim\varepsilon$ have very similar properties to those of the class *mu* enzymes, GST 7-7 and the class *alpha* enzymes of the rat liver cytosols, respectively, with respects to immunochemical reactions with the corresponding antibodies, substrate specificities and amino acid sequences [47].

GST 7-7 that does not exist at a detectable level in the normal rat liver cytosol but is markedly induced in the liver by the treatments of the animals with hepatocarcinogens [23, 48, 49], is one of the major GST isoenzymes in their kidney [50], lung [51], skeletal muscle [52], brain [53] and small intestine [54], although their GST 7-7 levels are very low compared with that of the hepatic preneoplastic tissue. (*S*)-STO was a better substrate than the (*R*)-enantiomer for GST 7-7; the ratio of K_{cat}/K_m for the (*R*)- to (*S*)-enantiomers was 1:2.4. That might be closely related to the previous demonstration that in the cytosol of rat small intestine, GSH conjugation of racemic STO took place more selectively towards its (*S*)-component while in the same rat liver cytosol, the (*R*)-component of the racemate was conjugated at a faster rate than the enantiomeric counterpart [55]. The rat small intestine has a relatively high level of GST 7-7 compared to those of the class *mu* enzymes [54].

There was a marked difference in regioselectivity between (*R*)- and (*S*)-STOs in their hepatic cytosolic GSH conjugations while little difference was observed in that of their non-enzymic GSH conjugations. No information has been obtained as yet of which isoenzyme(s) play(s) a part in increasing the ratio of the non-benzylic to the benzylic carbon conjugates in hepatic cytosolic GSH conjugation of (*S*)-STO.

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