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REGIOSPECIFIC AND DIASTEREOSELECTIVE INACTIVATION OF MUTAGENIC 9,10-DIHYDROBENZO[*a*]-PYRENE 7,8-OXIDE BY HEPATIC CYTOSOLIC GLUTATHIONE *S*-TRANSFERASE

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Abstract—Racemic, (7*R*,8*S*)-(+)-, and (7*S*,8*R*)-(−)-9,10-dihydrobenzo[*a*]pyrene 7,8-oxides (DBPOs) showed markedly different mutagenicity towards *Salmonella typhimurium* TA 98 in the order of (7*R*,8*S*)-(+)- > racemic > (7*S*,8*R*)-(−)-DBPOs. The enantiomeric epoxides were inactivated at significantly different rates by preincubating with rat liver cytosol fortified with glutathione (GSH) in the order of (7*S*,8*R*)-(−)- > racemic > (7*R*,8*S*)-(+)-DBPOs. Two non-mutagenic water-soluble metabolites were isolated from the preincubation mixture containing racemic DBPO as a substrate, separated by hplc, and identified by ^{13}C nmr and uv absorption spectroscopy as diastereoisomers of *S*-(8-hydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyren-7-yl)glutathione (conjugates I and II). Conjugates I and II were specifically yielded from (7*R*,8*S*)-(+)- and (7*S*,8*R*)-(−)-DBPOs, respectively, at different rates by rat liver cytosol; apparent values of K_m were 20.1 and 15.6 μM and of V_{max} 17.2 and 26.7 nmole/mg protein/min for (7*R*,8*S*)-(+)- and (7*S*,8*R*)-(−)-DBPOs, respectively. Conjugates I and II, therefore, were reasonably assigned to have (7*S*,8*S*)- and (7*R*,8*R*)-configurations, respectively. Conjugate II was yielded preferentially to conjugate I from racemic DBPO at an early stage of the enzymic reaction.

Glutathione (GSH) conjugation plays an important role in detoxifying a variety of electrophilic compounds, including active metabolites of carcinogens and mutagens [1–3]. Epoxides are among the carcinogenic or mutagenic nucleophiles that are conjugated with GSH by GSH *S*-transferase in various tissues in animals [4]. Although extensive studies have been made on the nature and the isozymes of the hepatic soluble transferase [5], only a little information is available on the reaction mechanism of the enzymic GSH conjugation of epoxides. This has been mainly attributable to difficulty in separation, purification and structural assignment of the peptide conjugates formed from the chiral epoxide molecule with two reactive carbons. However, recent progress in high performance liquid chromatography (HPLC) and Fourier transform ^{13}C NMR spectroscopy have made it possible to separate and characterize the diastereoisomers of the conjugates [6–11]. The first success in partial separation of the diastereoisomeric conjugates and assignment of their total structures has been achieved by means of these methods with regioisomeric GSH conjugates selectively formed from phenyloxiran (styrene 7,8-oxide) enantiomers [7,8]. Later, each pair of the regioisomeric conjugates was resolved by HPLC [12] although these data were contradictory to the previously reported ones in the benzylic to non-benzylic ratio of the conjugates formed.

Prior to the work on the conjugates of enantiomeric phenyloxiran, GSH conjugates of racemic benzo[*a*]pyrene 4,5-oxide, enriched with ^{13}C either at C_4 or C_5, had been demonstrated to be separable by HPLC into three components whose structures were partially assigned by ^{13}C NMR [13]. A recent study carried out with enantiomeric K-region epoxides of aza-arenes and arenes, including benzo[*a*]pyrene, has shown that they were all highly stereoselectively conjugated with GSH at their *R*-carbons [14].

It has been demonstrated with enantiomeric phenyloxirans that enzymic GSH conjugation of such an epoxide that has both benzylic and non-benzylic carbons in its oxiran ring does not necessarily occur specifically at the benzylic carbon in spite of its higher electrophilicity [7, 8].

However, substitution of the hydrogen atom at the non-benzylic carbon of phenyloxiran for a methyl group (racemic *trans*-2-methyl-3-phenyloxiran) results in the specific conjugation of GSH at the benzylic carbon [11, 12]. Similarly, regiospecific introduction of the GSH sulphydryl group to the benzylic oxiran carbon occurs with dihydrobenzarene oxides such as the olefinic epoxides of 1,2-dihydronaphthalene, 1,2-dihydrophenanthrene, and 7,8-dihydrobenzo[*a*]pyrene [11]. The mono-alkyl-substituted non-benzylic oxiran, 7-glycidoxycoumarin, is specifically conjugated with GSH at the less hindered oxiran carbon [10].

From the mechanistic view point, one of the most important problem remaining quite equivocal is on enantio-selectivity in enzymic GSH conjugation of epoxides of olefins. During the course of our investigation of the effect of GSH on mutagenicity of enantiomeric epoxides, it was found that the enantiomers of 9,10-dihydrobenzo[*a*]pyrene 7,8-oxide (DBPO) were inactivated at significantly different rates in the presence of rat liver cytosol [15]. The

present paper deals with the marked difference in intrinsic mutagenicity of the DBPO enantiomers towards *Salmonella* and with the stereochemical mechanism of their enzymic GSH conjugation.

MATERIALS AND METHODS

Materials. Racemic [16], (7*R*,8*S*)-(+)- and (7*S*,8*R*)-(−)-DBPOs [17] were synthesized as previously reported. Enantiomeric homogeneity of these epoxides was confirmed by determining their $[\alpha]_D$ values after derivatization from diastereoisomeric *l*-menthoxyacetates of *trans*-7-hydroxy-8-bromo-7,8,9,10-tetrahydrobenzo[*a*]pyrene [17] which were separated at retention times of 8.8 and 10.6 min by HPLC on a Nucleosil 50-7 silica column (7 μ, 30 cm × 4 mm) eluted with *n*-hexane containing 1% tetrahydrofuran (3 ml/min).

GSH, free from its oxidized form, was obtained from Yamanouchi Pharmaceutical Co. Ltd., Tokyo. The other reagents used were of reagent grade.

Preparation of liver cytosol. A post mitochondrial fraction obtained in isotonic KCl from a 3-volume liver homogenate of male Wistar rats (100–120 g) was centrifuged at 105,000 *g* for 1 hr. The supernatant fraction separated was dialysed at 2° for 20 hr through a Visking membrane tube against 2500 volumes of 0.1 M phosphate buffer, pH 7.4, and its volume was adjusted with the same buffer, so that 1 ml of the solution might contain 35 mg protein. Protein was determined by the method of Lowry *et al.* [18].

Mutagenicity test. The test was carried out according to the method of Ames *et al.* [19] with slight modification as follows: a solution of the epoxide in dimethyl sulphoxide (DMSO, 0.1 ml) was added to the dialysed soluble supernatant fraction of rat liver (20 μl), containing GSH (2.8 μmole), and incubated at 37° before mixing with 0.1 ml of an overnight culture of *Salmonella typhimurium* TA 98 (2×10^9 cells/ml) and top agar. The mixture was placed on a minimal agar plate for 2 days incubation at 37°. For testing intrinsic mutagenicity of the epoxides, the DMSO solution was directly added to the bacterial suspension in 0.1 M phosphate buffer, pH 7.4, mechanically agitated for 5 sec and then diluted with top agar.

GSH conjugation of DBPO. A typical incubation mixture consisted of the epoxide (80 nmole) dissolved in DMSO (80 μl), the dialysed soluble supernatant fraction (0.18 mg protein), and GSH (8 μmole) in a final volume of 2 ml of 0.1 M phosphate buffer, pH 7.4, and incubated at 37° for 20 min. After removing the unreacted epoxide by mechanical shaking twice with ether (5 ml each), GSH conjugates formed were quantitatively adsorbed on an Amberlite XAD-2 column (1 × 20 cm) and eluted with methanol as previously reported [8]. A residue obtained on evaporation of the solvent from the column effluent under reduced pressure was dissolved in water and subjected to HPLC performed under the conditions as described below.

Isolation of the GSH conjugates for recording ^{13}C NMR spectra was carried out by using a large scale of the incubation mixture (500 ml) consisting of DBPO (0.5 mM), the soluble supernatant fraction (3.5 mg protein/ml), and the same concentrations of the other ingredients as mentioned above and by using an Amberlite XAD-2 column (3 × 20 cm). The conjugates were obtained as amorphous solids on evaporating the solvent after being separated by HPLC, dissolved in water, and precipitated by adding methanol before recording the spectra.

Chromatography. HPLC of the GSH conjugates was carried out on a Constametric Model II G high performance liquid chromatograph equipped with a Shimadzu Model SPD-1 stop and flow spectrophotometer and a Nucleosil $7C_{18}$ octadecylsilicone (ODS) column (30 cm × 4 mm, 5 μ in particle size). The column was eluted with methanol–water–acetic acid (100:100:1), and the chromatograms were monitored at 280 nm unless spectra were recorded.

Thin-layer chromatography (TLC) was carried out on Merck F_{254} silica plates in *n*-butanol–acetic acid–water (4:1:2). The chromatograms were visualized as fluorescent spots with a u.v. lamp (254 nm) or as colouring spots with ninhydrin.

Spectroscopy. ^{13}C NMR spectra were recorded in D_2O on a JEOL Model TX-100 NMR spectrometer, and u.v. absorption spectra on a Hitachi Model 557 spectrophotometer.

Circular dichroism (CD). CD curves of the GSH conjugates were recorded at 20° in water on a JASCO Model J-500 C spectropolarimeter after being isolated by HPLC.

RESULTS

Mutagenicity of DBPO enantiomers and their inactivation by hepatic GSH S-transferase

(7*R*,8*S*)-(+)- and (7*S*,8*R*)-(−)-DBPOs with highly enantiomeric purity showed markedly different intrinsic mutagenicity towards *S. typhimurium* TA 98 strain bacteria. (7*R*,8*S*)-(+)-DBPO was three times as mutagenic as the (7*S*,8*R*)-(−)-isomer, and the mutagenicity of the racemate was between those of the enantiomers (Fig. 1A).

The mutagenicity of these epoxides rapidly decreased with difference in rate by preincubating for several minutes with a hepatic dialysed soluble supernatant fraction in the presence of GSH prior to exposure to the bacteria. (7*S*,8*R*)-(−)-DBPO had lost its mutagenicity almost completely 5 min after the preincubation whereas the mutagenicity of racemic and (7*R*,8*S*)-(+)-DBPOs was lost to a lesser relative extent and still remained at the time (Fig. 1B). GSH affected the mutagenic activities of the epoxides only to a slight extent when the soluble supernatant fraction was heated or omitted from the preincubation mixtures. The untreated soluble supernatant fraction also little affected their mutagenicity without GSH.

Regiospecific GSH conjugation of DBPO by hepatic GSH S-transferase

Two water-soluble, u.v.-absorbing, non-mutagenic metabolites were quantitatively isolated on an Amberlite XAD-2 column from the incubation mixture, consisting of racemic DBPO, the hepatic soluble supernatant fraction and GSH, and separated with remarkable difference in retention time, 38 (conjugate I) and 40 (conjugate II) min, on an ODS column by HPLC. They had the same u.v.-absorbing

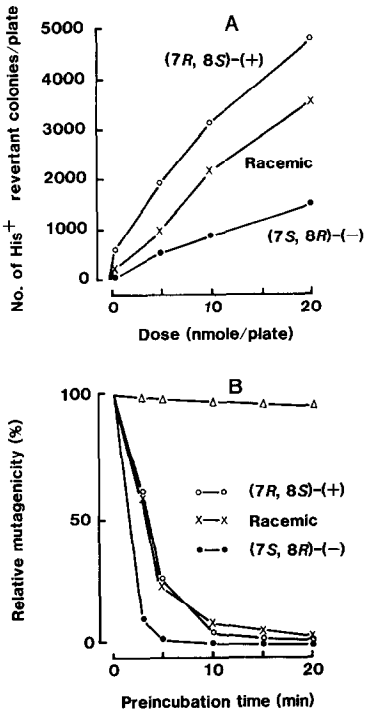
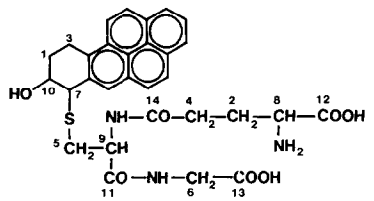


Fig. 1. Mutagenicity of enantiomeric and racemic DBPOs towards *S. typhimurium* TA 98 in the absence and in the presence of rat liver cytosol and GSH. (A) Bacteria were added to the solution of the epoxide, agitated and immediately diluted with top agar for further incubation on minimal agar plates. (B) Bacteria were added to the preincubation mixture, consisting of the epoxide (40 nmole), dialysed soluble supernatant fraction (1.0 mg protein/ml) of rat liver homogenate and GSH (4 mM), and worked up as mentioned above. Data are mean values of six experiments. △–△ represents relative mutagenicity of the three epoxides (no appreciable difference) exerted when the soluble supernatant fraction was omitted or boiled.

peak areas in the HPL-chromatogram when incubated until the epoxide was completely consumed (80 min). From the results of incubation with a boiled soluble supernatant fraction, approximately 6% of the GSH conjugates were found to be non-enzymically formed under the same conditions. The metabolites, separately eluted from the ODS column, showed single, ninhydrin-positive spots at the same R_f value in the thin-layer chromatogram and also showed completely superimposable u.v. absorption spectra; $\lambda_{max}^{H_2O}$ nm (ε): 345 (25,660), 329 (19,370), 315 (7700, shoulder), 281 (21,600), 270 (18,700) and 250 (43,600). Their ^{13}C NMR spectra were also superimposable on each other, and all the ^{13}C signals, appearing well resolved, were assignable (Fig. 2). The spectra indicated that the benzylic carbon C_7 was the only carbon bearing the sulphur atom of GSH since no ^{13}C signal appeared at the magnetic field ranging from 35 to 45 ppm where a ^{13}C signal due to a non-benzylic thioalkyl methine carbon, if present, would appear [6, 8]. Thus, it is postulated from the ^{13}C NMR spectroscopic data that the isolated conjugates were diastereoisomers of *S*-(8-hydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyren-7-yl)-glutathione.



Chemical shifts (ppm)

C		C	
1	28.3	9	56.3
2	29.5	10	71.4
3	31.5	11	173.0
4	32.5	12	175.7
5	34.6	13	176.5
6	44.9	14	177.2
7	52.6	aromatic	123.4 – 132.3
8	55.7		

Fig. 2. ^{13}C NMR spectra of GSH conjugates enzymically formed from enantiomeric and racemic DBPOs. The spectra were recorded in D_2O as 170 mM solutions. The conjugates were isolated on an Amberlite XAD-2 column, separated by HPLC and precipitated from water by adding methanol.

(7*R*,8*S*)-(+)-DBPO yielded the fast-eluting GSH conjugate (I) without concomitant formation of any trace amount of its isomer when incubated under the same conditions until the epoxy substrate was completely consumed. On the other hand, (7*S*,8*R*)-(−)-DBPO specifically yielded the slow-eluting GSH conjugate (II) alone when incubated under the same conditions (Fig. 3). Thus, it was concluded that con-

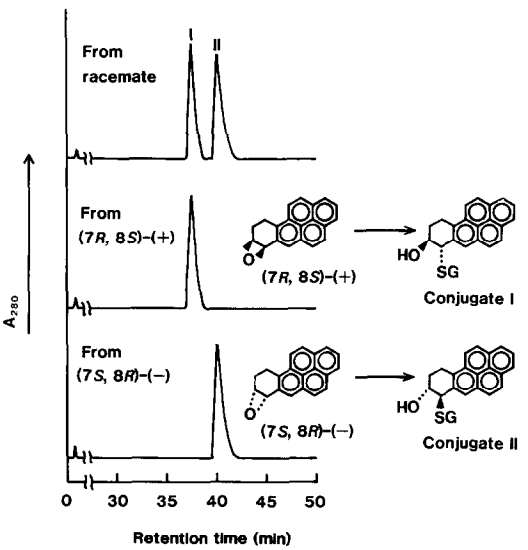


Fig. 3. HPLC of GSH conjugates enzymically formed from racemic and enantiomeric DBPOs. The epoxides (0.04 mM each) were completely conjugated by incubation for 80 min with hepatic soluble supernatant fraction (0.09 mg protein/ml) in the presence of GSH (4 mM). The conjugates were isolated from the incubation mixtures by adsorption on an Amberlite XAD-2 column and eluted with methanol. HPLC conditions: column—Nucleosil $7C_{18}$ (5 μ in particle size, 30 cm × 4 mm); developing solvent—MeOH:H_2O:CH_3COOH (100:100:1, 1.5 ml/min).

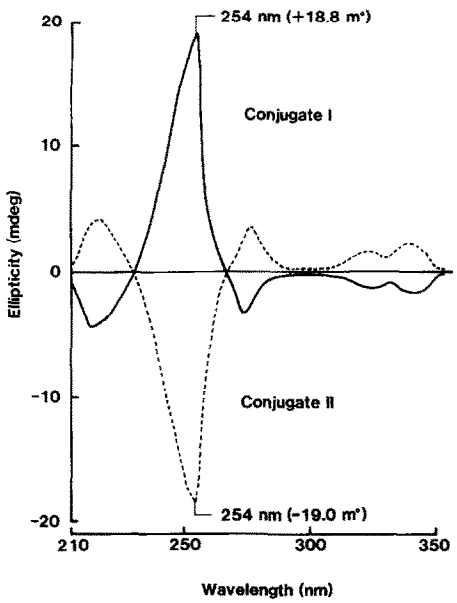


Fig. 4. Circular dichroism curves of GSH conjugates enzymically formed from enantiomeric and racemic DBPOs. The conjugates were isolated and separated as described in Fig. 3. The curves were recorded at 20° in aqueous solutions of the conjugates (0.02 mM each).

jugate I from (7*R*,8*S*)-(+)-DBPO had the (7*S*,8*S*)-configuration and that conjugate II from (7*S*,8*R*)-(−)-DBPO had the (7*R*,8*R*)-configuration. CD curves of these conjugates supported the conclusion on the configuration of their C_7 and C_8. The (7*S*,8*S*)-conjugate (I) showed a (+)-CD curve with peak maximum at 254 nm, and the (7*R*,8*R*)-conjugate (II) a (−)-CD curve with the same magnitude of elliptic amplitude in the wavelength region ranging from 220 to 350 nm (Fig. 4). The same molar concentration of

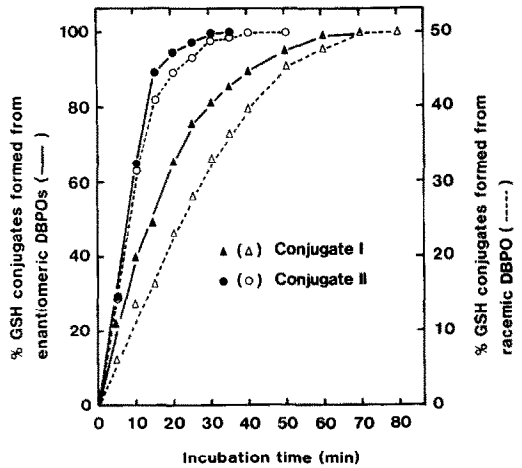


Fig. 5. A comparable time course study of enzymic GSH conjugation of enantiomeric and racemic DBPOs. The epoxides (0.04 mM each) were incubated at 37° with hepatic dialysed soluble supernatant fraction (0.09 mg protein/ml) in the presence of GSH (4 mM). The conjugates formed were isolated and determined by HPLC as described in Fig. 3.

GSH showed no detectable CD in this wavelength region.

The same diastereoisomeric conjugates were also specifically obtained in almost quantitative yield by the reaction of the respective epoxides (126 mM each) with GSH (190 mM) in methanol, tetrahydrofuran and 5% aqueous sodium bicarbonate (1:1:1) at room temperature for 3 hr and identified with the metabolites by chromatography, spectroscopy and CD; conjugate I was yielded from (7*R*,8*S*)-(+)-DBPO, conjugate II from (7*S*,8*R*)-(−)-DBPO, and both of them in the equal ratio from the racemic DBPO.

Enantioselectivity in GSH conjugation of DBPO by hepatic GSH S-transferase

As assumed from the profile of the inactivation of the mutagenic DBPO enantiomers by the hepatic soluble supernatant fraction in the presence of GSH (Fig. 1), the (7*S*,8*R*)-epoxide was conjugated more readily than the (7*R*,8*S*)-epoxide when separately incubated with the soluble supernatant fraction. Kinetic experiments carried out by using HPLC indicated that apparent K_m and V_{max} were 20.1 μM and 17.2 nmole/mg protein/min for (7*R*,8*S*)-(+)-DBPO and 15.6 μM and 26.7 nmole/mg protein/min for (7*S*,8*R*)-(−)-DBPO, based on the double reciprocal plots obtained from the GSH conjugates formed vs concentrations of enantiomeric DBPOs (1–40 μM each).

A comparable time course study on the enzymic GSH conjugation reactions of enantiomeric and racemic DBPOs showed that the formation of conjugate I yielded from the (7*R*,8*S*)-component in racemic DBPO was significantly retarded compared with that from (7*R*,8*S*)-(+)-DBPO alone (Fig. 5); the former was approximately one half of the latter in the zero-order kinetics region of the reaction. On the contrary, the formation of the conjugate II from the (7*S*,8*R*)-component in racemic DBPO was retarded only a little compared with that from (7*S*,8*R*)-(−)-DBPO alone.

DISCUSSION

Potent mutagenicity to *Salmonella* has been reported on a variety of racemic olefinic epoxides of dihydrobenzarenes, including DBPO [20]. Only a few attempts, however, have been made to confirm the difference of epoxide enantiomers in mutagenicity to bacteria. To our knowledge, benzo[*a*]-pyrene 4,5-oxide [21], 3,4-epoxy-1,2,3,4-tetrahydrochrysene [22], 1,2- and 3,4-epoxy-tetrahydrobenz[*a*]anthracenes [23], and phenyloxiran [24] are the cases that the enantiomers showed higher mutagenicity to *Salmonella* than their counterparts. It is of interest that the enantiomers with *R*-benzylic carbon show higher mutagenicity towards the TA 98 strain bacteria than the counterparts with *S*-benzylic carbon so far as these olefinic epoxides, including DBPO, are concerned.

In spite of its much lower intrinsic mutagenicity to TA 98 than (7*R*,8*S*)-(+)-DBPO, the remaining activities of racemic DBPO were almost the same as those of the enantiomer after the preincubation with hepatic cytosol in the presence of GSH (Fig. 1B).

This would be attributable to the retarded conjugation of the more mutagenic component, (7*R*,8*S*)-(+)-DBPO, in the racemate. (7*S*,8*R*)-(−)-DBPO seems to have higher affinity for hepatic GSH *S*-transferase than the (7*R*,8*S*)-enantiomer so far as estimated from their K_m values and to inhibit enzymic conjugation of the counterpart. Actually, the rate of enzymic formation of conjugate I from the slow-reacting, more mutagenic, low affinity component, (7*R*,8*S*)-(+)-DBPO, in the racemate was shown by the time course study to be considerably retarded compared to the conjugation of the enantiomer alone (Fig. 5). On the contrary, the time course study indicated that the rate of conjugation of the fast-reacting, less mutagenic, high affinity component, (7*S*,8*R*)-(−)-DBPO, was only slightly inhibited by the counter epoxide in the racemate. For further consideration of the data of the metabolic inactivation, it should be noted that enzymic inactivation of the epoxides kept going on after diluting the preincubation mixtures with soft agar for further incubation on hard agar plates. Consequently, it is readily suggested from the time course study of the enzymic conjugation reaction that the more mutagenic component in racemic DBPO is likely to remain in the increasing ratio to the less mutagenic counterpart during the course of the incubation on the hard agar plates.

Only from the viewpoint of nucleophilicity, it seems to be reasonable that the sulphydryl group of GSH makes a sulphide bonding specifically to the benzylic oxiran carbon of DBPO in the enzymic conjugation reaction as well as to that of the very recently reported olefinic epoxides of a series of dihydrobenzarenes [11], since the same regiospecificity occurs non-enzymically on the reaction of the epoxides with GSH at alkaline pH. As for DBPO, ethyl and *t*-butyl mercaptans also attack specifically on the benzylic oxiran carbon in the same alkaline medium to yield *trans*-α-hydroxy-alkyl sulphides without concomitant formation of their *cis*-isomers as far as checked by silica and ODS HPLC, CD, and ^{1}H NMR (data not shown).

Regiospecific introduction of the sulphydryl group of GSH to benzylic oxiran carbons of DBPO enantiomers was elucidated by ^{13}C NMR spectroscopy of the conjugates after they were isolated by HPLC as single products from the respective epoxides. The ^{13}C signals due to the *S*-bearing carbon and the carbinol carbon of their tetrahydronaphthalene nucleus appeared at 52.6 and 71.4 ppm, respectively. The NMR data are in good accordance with the previously reported ones on the enzymically or non-enzymically formed GSH conjugates of the olefin oxides of *trans*-β-methylstyrene, 1,2-dihydronaphthalene,3,4-dihydrophenanthrene, and 7,8-dihydrobenzo[*a*]pyrene, all of which show ^{13}C signals at the two different magnetic fields ranging from 47 to 60 ppm and 70 to 73 ppm for the benzylic thioalkyl methine carbons and the non-benzylic carbinol methine carbons, respectively [11]. Evidence was also obtained from the NMR spectra for the absence of a regioisomer with non-benzylic thiolkyl methine carbon in the DBPO–GSH conjugates isolated by HPLC. That is, the regioisomer, if present, could show ^{13}C signal due to the thioalkyl methine carbon detectable at a higher magnetic field ranging from 35 to 45 ppm as previously demonstrated with the chromatographically separated regioisomers of phenyloxiran–GSH conjugates; at 40.2 and 53.7 ppm appear the ^{13}C signals due to their non-benzylic and benzylic thioalkyl carbons, respectively [6, 8]. Furthermore, benzylic carbinol methine carbons, if present in the DBPO–GSH conjugates, would show ^{13}C signals at 65–70 ppm as previously demonstrated with 1-hydroxy-1,2,3,4-tetrahydronaphthalene and its 3-methyl derivatives [25]. No detectable signal, however, was found at this magnetic field in the ^{13}C NMR spectra of the DBPO conjugates.

The present data on the time course of enzymic conjugation of racemic DBPO may suggest that GSH conjugation of DBPO enantiomers is catalysed by a single transferase although the previous workers have demonstrated the participation of a few transferase isozymes in GSH conjugation of polynuclear arene oxides [1–3]. Noticeable data have recently been provided by Cobb *et al.* [14] from their study on GSH conjugation of the K-region epoxides of polynuclear arenes and aza-arenes. They showed the *R*-carbon of the epoxide enantiomers to be conjugated with high stereoselectivity by an isozyme of rat liver cytosolic GSH *S*-transferases.

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