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SHORT COMMUNICATIONS

Regiospecific glutathione conjugation of alkylarylethylene oxides by hepatic glutathione *S*-transferase

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Glutathione (GSH) conjugation plays an important role in detoxication of a variety of reactive metabolites of toxic or carcinogenic xenobiotics [1–3]. A remarkable progress has recently been made in the enzymological study on GSH *S*-transferase [4]. Despite its importance in detoxication, however, only a little information has been available on the mechanism of GSH conjugation of epoxides, especially on regioselectivity and enantioselectivity in the introduction of the SH group of GSH to their oxiran carbons. Enantiomeric k-region epoxides of arenes [5, 6] and azaarenes [6] have been demonstrated to be highly regioselectively conjugated with GSH by hepatic soluble GSH *S*-transferase. Apart from these epoxides with two benzylic carbons in their oxiran rings, only an instance has been reported with phenyloxiran (styrene 7,8-oxide) [7, 8] for enzymic regioselective GSH conjugation of olefinic epoxides having a benzylic and a non-benzylic carbons in the oxiran ring. This type of epoxides are of importance because they include diol-epoxides of carcinogenic polynuclear aromatic hydrocarbons. Enzymic GSH conjugation of benzo[*a*]pyrene-7,8-diol-9,10-epoxide has recently been reported [9]. However, it is still equivocal with this olefinic epoxide which oxiran carbon makes a sulphide bonding with GSH. In the case of phenyloxiran, based on evidence for ^{13}C NMR spectroscopy of the chromatographically isolated conjugates, it was demonstrated that the *R*- and *S*-enantiomers are almost specifically conjugated at the benzylic and non-benzylic carbons, respectively. The present communication deals with regiospecific GSH conjugation of carcinogenic or mutagenic alkylarylethylene oxides at their benzylic oxiran carbons by rat liver cytosolic GSH *S*-transferase.

Racemates of phenyloxiran **1**, *trans*-1-phenyl-2-methyl ethylene oxide (*trans*-β-methylstyrene oxide) **2** [10], 1,2-epoxy-1,2,3,4-tetrahydronaphthalene **3** [11], 3,4-epoxy-1,2,3,4-tetrahydrophenanthrene **4** [12], and 9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene **5** [13] were separately incubated with rat liver cytosol in the presence of GSH as previously reported [7, 8]. From the mixture was collected the GSH conjugate on an Amberlite XAD-2 column as has been reported with phenyloxiran-GSH conjugates [7, 8], following removal of the unreacted substrate by extraction and of coagulated protein by filtration.

The conjugate from each epoxide, eluted from the column with aqueous methanol, showed a single, ninhydrin-positive and u.v.-absorbing spot on cellulose thin-layer chromatograms as well as on silica gel plates obtained in an admixture of *n*-butanol–acetic acid–water (4:1:1). The conjugates in the XAD-2 column eluates were subjected to reverse partition HPLC on an octadecylsilicone column for further resolution and purification. As had been demonstrated, the conjugates of phenyloxiran **1** appeared as well separated double peaks in the peak area ratio 3:2 in the HPL chromatogram monitored with a u.v. detector, and they were identified as the regioisomers, *S*-(1-phenyl-2-hydroxyethyl)GSH **1'A** and *S*-(2-phenyl-2-hydroxyethyl)-GSH **1'B**, with the corresponding authentic specimens [8]. The conjugates of the other epoxides, however, showed single peaks on the same HPLC column in analogous solvent mixtures (Fig. 1). The peak width of conjugate **3**, however, was somewhat broader than that of the others. U.v. absorption spectra in aqueous methanol of the conjugates eluted from the HPLC column showed that they had the same u.v.-absorbing chromophors as those of the corresponding epoxides.

^{13}C-Signals arising from aliphatic carbons in the peptide and acyclic or cyclic alkyl side chains attached to the aromatic nuclei of the conjugates appeared well resolved at 22.9 to 72.8 ppm in the NMR spectra and were all assignable by using an off-resonance technique. The NMR spectra indicated the chromatographically isolated conjugates **2'**,

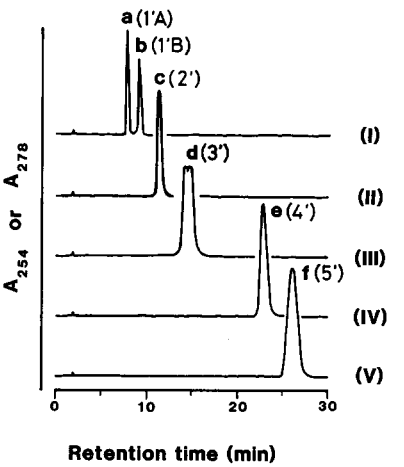
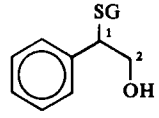
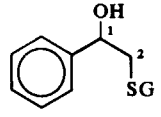
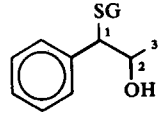
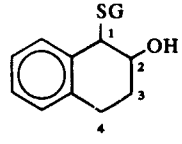
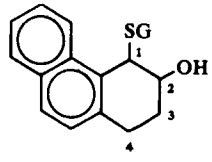
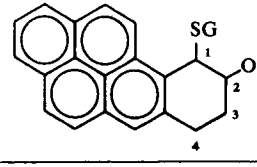


Fig. 1. HPLC-separation of GSH conjugates formed during incubations of phenyloxiran, *trans*-β-methylstyrene oxide, 1,2-epoxy-1,2,3,4-tetrahydronaphthalene, 3,4-epoxy-1,2,3,4-tetrahydrophenanthrene, and 9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene with a GSH-fortified cytosolic fraction from rat liver. The epoxides (2 mM except for **5** (0.2 mM)) were incubated at 37° for 7 min (5 min for **5**) with a cytosolic fraction (1 mg protein/ml except for **5** (7 mg protein/ml)) from liver of male Wistar rats (100–120 g) in 0.1 M phosphate buffer, pH 7.4, containing acetone (2%, v/v). The substrate was dissolved in the acetone and added to the mixture that was preincubated for 10 min. Termination of the reaction and quantitative isolation of the conjugates were carried out as previously reported [7, 8]. A Nucleosil $7C_{18}$ column was used (3.9 mm × 30 cm) and developed with 25% (I–III), 30% (IV), or 55% (V) MeOH and 0.5% acetic acid in H_2O as eluants at a flow rate of 1.0 ml/min. The chromatograms were monitored at 254 nm (I–IV) or 278 nm (V). Peak a: *S*-(1-phenyl-2-hydroxyethyl)-GSH **1'A**; peak b: *S*-(2-phenyl-2-hydroxyethyl)GSH **1'B**; peak c: *S*-(1-phenyl-2-hydroxypropyl)GSH **2'**; peak d: 1-*S*-glutathionyl-2-hydroxy-1,2,3,4-tetrahydronaphthalene **3'**; peak e: 4-*S*-glutathionyl-3-hydroxy-1,2,3,4-tetrahydrophenanthrene **4'**; peak f: 10-*S*-glutathionyl-9-hydroxy-7,8,9, 10-tetrahydrobenzo[*a*]pyrene **5'**.

Table 1. ^{13}C NMR spectra of GSH conjugates formed from olefin epoxides by GSH *S*-transferase

GSH conjugate*		Chemical shifts (ppm)†											
		C_1	C_2	C_3	C_4	Aromatic C	Carbonyl C	Cys-C_α	Glu-C_α	Gly-C_α	Cys-C_β	Glu-C_γ	Glu-C_β
1'A		53.7	65.3	—	—	129.6 −126.9	175.0 −173.0	53.7	52.3	42.6	33.2	31.9	26.8
1'B		73.1	40.2	—	—	129.4 −126.7	175.0 −172.7	53.7	52.0	44.3	33.9	31.8	26.6
2'		59.9 59.4	72.8	22.9	—	141.9 −130.5	178.8 −174.2	56.9	56.7	46.1	35.4	34.2	29.2
3'		52.6 51.6	71.9 71.6	26.7 26.4	28.1 27.7	139.5 −129.0	178.8 −174.4	56.9	56.3 55.8	46.2	35.5	34.2	29.1
4'		48.3 46.9	71.6 70.7	25.7	26.7	138.0 −125.8	178.8 −174.3	56.9	56.5 56.0	46.9	35.9	34.2	29.0
5'		53.7‡	71.5‡	24.9	31.7	135.1 −123.2	176.0 −173.0	56.5	55.1‡	44.2	32.5	29.3	27.3

* Conjugates (200 mM for **1'–4'** and 50 mM for **5'**) were dissolved in D_2O.
† Chemical shifts are expressed in ppm from TMS (upper two conjugates) and from TSP (lower four) signals.
‡ Broad singlet.

3', **4'**, and **5'** from the respective epoxides **2**, **3**, **4**, and **5** to be homogeneous in view of regioisomerism since the ^{13}C-signals from >CH–SG and >CH–OH of the alkyl side chains appeared at 46.9–59.9 and 70.7–72.8 ppm, respectively (Table 1). The ^{13}C-signals of **2'**C_1, **3'**C_{1-4}, **4'**C_{1-2}, **3'**Glu C_α, and **4'**Glu C_α appeared as doublets in the relative integrated peak ratio 0.5:0.5 with very small difference in chemical shift. Occurrence of doublet ^{13}C-signals has been observed with >CH–OH and >CH–SG of benzo[*a*]pyrene 4,5-oxide–GSH conjugates [5] and with >CH–OH of 7-glycidoxycoumarin–GSH conjugate [14]. The ^{13}C NMR data postulate that in the conjugate molecules, the glutathionyl *S*-bearing carbon is located at the benzylic position and the carbinol carbon at the non-benzylic one. The signals from non-benzylic ^{13}C–SG and benzylic ^{13}C–OH, if present as contaminants, would appear at 35–45 ppm and 60–75 ppm in the spectra, respectively. The ^{13}C NMR data, however, showed the conjugates to be free from the regioisomers with the non-benzylic glutathionyl *S*-carbon in the alkyl chains. The GSH conjugates from the racemic epoxides used in the present investigation all consist of two possible diastereomers which could not be resolved by HPLC on several ODS columns and solvent mixtures examined. Only conjugate **3** showed a broad and slightly resolved double peak with a very small difference in retention time which we failed to separate even when the conjugates were eluted by more polar solvent systems under the conditions similar to those described in Fig. 1.

Thus, racemic alkyloxirans directly linking to aromatic rings are regiospecifically conjugated with GSH at the benzylic oxiran carbon by rat liver cytosolic GSH *S*-transferase as summarized in Fig. 2. The regiospecificity in GSH conjugation would be attributable to a steric hindrance effect of the methyl or cyclic alkyl group in the epoxide molecule on introduction of the SH group of GSH to the non-benzylic carbon because 7-glycidoxycoumarin [14], a very recently proposed fluorophotometric substrate for epoxide–GSH transferase, for example, has been demonstrated to be specifically conjugated with GSH at the less hindered non-substituted oxiran carbon of its two non-benzylic ones.

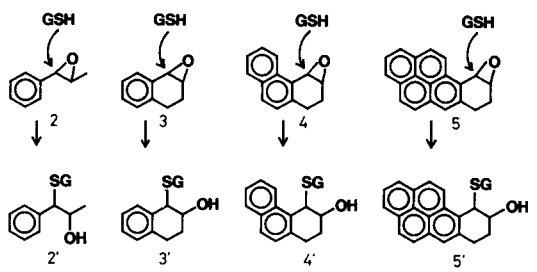


Fig. 2. Regiospecific conjugation of GSH at the benzylic oxiran carbon of racemic alkylarylethylene oxides by the catalytic action of GSH *S*-transferase.

However, it should be emphasized that the epoxides used in the present investigation are also regiospecifically conjugated with GSH at the benzylic position in the absence of the hepatic cytosol. The epoxides (2 mM except for **5** (0.2 mM)) reacted with GSH (4 mM) at much slower rates than those in the enzymic reaction; the non-enzymic conjugation rates were 6.2, 7.1, 20.5, 43.7, and 1.1 μM/min for **1**, **2**, **3**, **4**, and **5**, respectively, at pH 7.4 and 37°. However, at pH 10, they formed the benzylic conjugates rapidly in quantitative yield within 30 min at 37°, which were identified with the enzymically formed conjugates by HPLC, TLC, u.v. absorption and ^{13}C NMR spectroscopy. Reactions of these epoxides with *t*-butyl mercaptan as well as ethyl mercaptan at the alkaline pH also resulted in the formation of single products with the alkyl sulphides specifically at their benzylic positions, which were identified by ^{1}H NMR, u.v. absorption, and mass spectroscopy. This fact implies that a factor determining direction of the attack of SH compounds to these epoxides depends not only on a steric hindrance effect of the alkyl substitutions, but also on an additional effect of higher electrophilicity of the benzylic oxiran carbons.

Relative reaction rates of enzymic GSH conjugation of these epoxides (non-enzymic reaction rates subtracted) under the aforementioned conditions were 1, 1.04, 0.88, 0.69, and 0.45 for **1**, **2**, **3**, **4**, and **5**, where the relative rate 1 for **1** was based on the sum of the regioisomeric conjugates **1'A** and **1'B** and represents 96.8 nmole/mg cytosolic protein/min. The order of the observed rates for enzymic GSH conjugation of arene-fused cyclohexene oxides were in accordance with the previously reported data on the corresponding arene oxides which were demonstrated by Hayakawa *et al.* [15] with a sheep liver GSH *S*-transferase preparation, based on selective adsorption of [^{35}S]GSH conjugates on a charcoal column.

The structure of the GSH conjugate **3'** from 1,2-epoxytetrahydronaphthalene **3**, has previously been proposed by Booth *et al.* [16], based on the fact of 2-naphthol formation from **3'** by the treatment with Raney nickel. They identified the naphthol by paper partition chromatography. Although they had provided the same structural evidence as that of the present investigation, it should be noticed that 2-naphthol may arise not only from **3'** but also from its non-benzylic regioisomer as has previously been demonstrated with catalytic desulphuration of **1'A** and **1'B** under the same conditions [17].

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Effects of monocrotaline treatment on norepinephrine removal by isolated, perfused rat lungs

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Monocrotaline (MCT) is a toxic pyrrolizidine alkaloid isolated from the leaves and foliage of the *Crotalaria spectabilis* [1]. Administration of MCT to rats results in the development of increased pulmonary arterial pressure, medial thickening in the pulmonary vasculature, and right ventricular hypertrophy [2–4]. In addition, pulmonary capillary endothelial cells show morphological and functional signs of injury. The cells swell and protrude into the vessel lumen and have increased numbers of cytoplasmic organelles and pinocytotic vesicles, as well as enlarged nuclei [5]. These changes are similar to those observed in patients with primary pulmonary hypertension [6], suggesting that MCT-induced pulmonary hypertension in rats might be a useful model for the human disease.

Endothelial cells are the site of a specific, carrier-mediated uptake process for 5-hydroxytryptamine (5-HT) [7, 8]. Monocrotaline treatment decreases the removal of perfused 5-HT by isolated rat lungs [9, 10]. Another biogenic amine, norepinephrine (NE), is also taken into pulmonary endothelial cells by a saturable, carrier-mediated process [7, 11, 12]. Although similar to the 5-HT uptake process, NE uptake seems to occur at pharmacologically different sites [12]. Previous reports of the effects of MCT on NE uptake have presented apparently conflicting observations. One group [9] reported a marked decrease in the NE removal by isolated lungs of rats perfused at 37° after 3 weeks of MCT feeding, but this conclusion was based on a small number of observations (N = 3). Another group [10] using the same treatment regimen detected no changes in NE transport by lungs perfused at room temperature. It would be of interest to know whether MCT does indeed affect NE uptake since human patients with elevated pulmonary vascular resistance have significantly reduced NE removal across the pulmonary circulation [13, 14] and increased plasma NE concentrations [15].

The purpose of this study was to determine whether a single dose of MCT, known to cause pulmonary hypertension, affects NE removal in the isolated, perfused rat lung preparation.

Male, Sprague–Dawley rats (Spartan Farms, Haslett, MI) were used for all experiments. The animals were housed on corn cob derived bedding and allowed free access to food (Wayne Lab-Blox, Continental Brain Co., Chicago, IL) and water. An alternating 12 hr light/dark cycle was maintained. Rats weighing 200–225 g were given a single subcutaneous injection of 105 mg MCT/kg or 0.9% saline. The MCT was dissolved in 0.2 N HCl, neutralized with NaOH, and then brought to volume to provide a working solution of 60 mg MCT/ml. Fourteen days after treatment, NE uptake and metabolism and right ventricular hypertrophy were evaluated.

The isolated, perfused lung preparation has been described in detail previously [3, 9, 16]. Briefly, rats were anesthetized with pentobarbital and treated with 500 units of heparin intravenously. The trachea and the pulmonary artery were cannulated, and the heart was cut away at the level of the atria. The lungs were carefully removed and transferred to a 37° chamber. The perfusion medium was pumped through the lung in a single pass system at a constant flow of 10 ml/min. The perfusion medium consisted of a Krebs–Ringer bicarbonate buffer (pH 7.4) aerated with 95% O_2/5% CO_2 containing 4% bovine serum albumin (Fraction V, Miles Biochemicals, Elkhart, IN) and 4.6 μM calcium disodium edetate.

The airways were filled with 2 ml of room air, and the tracheal cannula was clamped to keep the lungs statically inflated. Inflow perfusion pressure was monitored with a Statham P23ID pressure transducer and recorded on a Grass model 7 Polygraph. After the vasculature was cleared of blood, the perfusion medium was switched to one containing 0.1 μM [^{14}C]norepinephrine (DL-[8-^{14}C]noradrenaline DL-bitartrate, sp. act. 55 mCi/mmole, Amersham, Arlington Heights, IL). Effluent samples were collected 7.5 min after the introduction of [^{14}C]NE. At one end of perfusion, the lungs were removed from the apparatus, blotted, and immediately weighed. Radioactivity in aliquots of perfusion medium was determined directly and after separation on Biorex 70 (pH 6.0) columns. The deaminated metabolites were eluted with water, the unchanged amine was removed by elution with 2% boric acid, and then the O-methylated metabolites were eluted with 0.2 N HCl [17]. Percent removal of NE and percent of perfused NE appearing in the effluent as metabolites were calculated as previously described [3]. Removal of NE is defined as the difference between the concentration of unchanged NE in the inflow perfusion medium and that in the collected effluent. Percent metabolites are expressed as the percentage of perfused NE appearing as metabolites in the effluent perfusate.

Right ventricular hypertrophy was used to confirm the development of pulmonary hypertension. After removing the atria, the right ventricle (RV) was trimmed away, leaving the left ventricle plus septum (LV+S) intact. Each piece was weighed and the ratio RV/(LV+S) was calculated. An increase in RV/(LV+S) in the absence of changes in (LV+S) weight reflects right ventricular hypertrophy [18].

The effects of a single dose of MCT 14 days after treatment are summarized in Table 1. The development of pulmonary hypertension was indicated by right ventricular hypertrophy [an increase in the RV/(LV+S) ratio] and by an increase in the baseline perfusion pressure in the isolated lung preparation. The increased pressure is presumed to reflect an increase in the pulmonary vascular resistance due to medical thickening of the pulmonary blood vessels. The