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Glutathione *S*-conjugates of phenyloxirane

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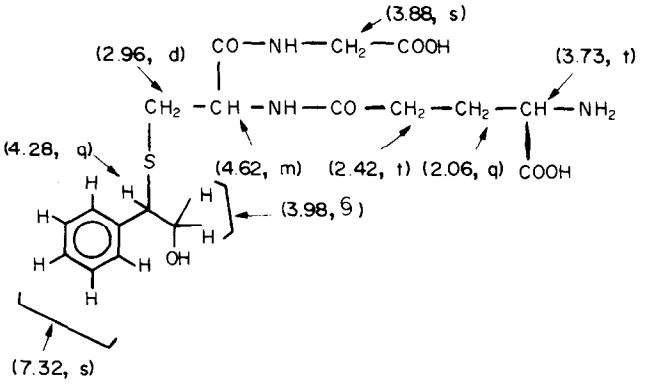
A variety of biochemical, pharmacological, and toxicological studies on glutathione *S*-conjugation of epoxides of xenobiotic olefins and arenes suggest that it plays an important role in the metabolic inactivation of toxic epoxides as active metabolites formed from parent hydrocarbons by microsomal monooxygenases. Phenyloxirane (styrene oxide), a mutagenic metabolite of the plastic monomer, styrene [1–3], has been frequently used as a standard substrate for the study of glutathione *S*-transferase [4–7]. In rat liver, the epoxide formed from styrene by microsomal monooxygenase is detoxicated through glutathione conjugation by cytosolic glutathione *S*-transferase at extremely high rate as well as through hydrolysis to phenylethanediol by microsomal epoxide hydratase at a considerably minor rate [8, 9].

However, no conclusive evidence has been available yet for the mode of introduction of the glutathione sulfhydryl group to the oxirane carbons of phenyloxirane in the metabolic conjugation reaction. Two approaches have recently been made to this problem, besides the earlier finding by James and White [10] that both styrene and phenyloxirane yielded *N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)cysteine *in vivo* and *in vitro* as the sole sulfur-containing metabolite. One was made by Ryan and Bend [8] who claimed the conjugate formed from phenyloxirane in rat liver to be single and identical with the sole product, *S*-(1-phenyl-2-hydroxyethyl)glutathione 1 synthesized from glutathione and phenyloxirane in an aqueous ethanolic sodium hydroxide solution. The other was made by Pachecka *et al.* [11] who reported that the synthetic conjugate of Ryan and Bend as well as a biologically formed glutathione conjugate from phenyloxirane in a cytosolic fraction of rat liver contained another isomer, *S*-(2-phenyl-2-hydroxyethyl)-glutathione **2**, in higher yield. Pachecka *et al.* isolated the biologically formed conjugates as an inseparable mixture on a silica gel column, subjected it to hydrogenolysis with Raney nickel in absolute ethanol, identified two isomeric phenylethanols as desulfuration products with phenethyl alcohol and methylphenylcarbinol, and emphasized that their results were coincident with those of the previous *in vivo* work done by Seutter-Berlage *et al.* [12]. The *in vivo* work, not necessarily providing direct evidence for the glutathione conjugation problem, showed that the rats given styrene excreted two isomeric phenylhydroxyethyl-mercapturic acids in urine which were readily separable on silica gel after derivatization with diazomethane and identified with synthetic specimens prepared from phenyloxirane and *N*-acetylcysteine methyl ester in an aqueous ethanolic sodium carbonate solution.

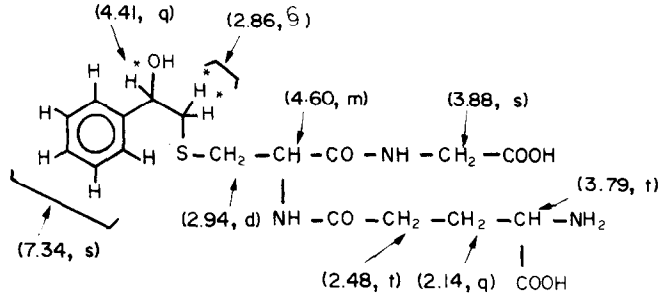
In the present communication, we wish to provide direct evidence for the formation of two isomeric glutathione conjugates **1** and **2** from phenyloxirane in rat liver cytosol. The conjugates were isolated, separated by h.p.l.c. (high performance liquid chromatography), and identified with respective synthetic specimens through the present work. Evidence will also be provided that the Raney nickel method reported by the previous workers be inadequate for the assignment of the conjugate structures since it yields both phenethyl alcohol and methylphenylcarbinol either from **1** or from **2** alone through phenyloxirane formed as a common intermediate by their desulfuration.

A mixture of glutathione conjugates **1** and **2** was prepared from phenyloxirane and isolated as a mixture on an Amberlite XAD-2 column by the method of Ryan and Bend. An amorphous solid isolated showed a single spot at R_f 0.40 on a microcrystalline cellulose powder plate containing a fluorescent reagent in *n*-butanol–water–acetic acid (4:1:1) as a developing solvent; the spot was visualized by u.v.-rays as well as by spraying with ninhydrin followed by heating. The conjugates were separated on an octadecylsilicone column (μBondapak C_{18}, 10 μ in particle size, 3.9 mm × 30 cm) in a solvent mixture of methanol–water–acetic acid (20:180:1) by h.p.l.c. at a column temperature of 30° with monitoring at 254 nm. The u.v.-absorbing peak areas in the chromatogram indicated that the synthetic conjugate mixture contained **1** and **2** in the ratio 1:3. Evaporation of the solvent from the eluate containing each conjugate, followed by recrystallizations from methanol–water, gave colorless prisms. Structures of the separated conjugates were assigned by proton-n.m.r. spectra (Fig. 1) by the partial application of the double resonance method.

In order to obtain biologically formed glutathione conjugates of phenyloxirane, a solution of the epoxide (2 mM) in aceton (270 mM) was incubated at 37° for 10 min in the presence of glutathione (4 mM) with a 105,000 *g* supernatant fraction (0.62 mg protein/ml) from a young adult male Wistar rat liver homogenate in 0.1 M phosphate buffer, pH 7.4. The biological reaction was terminated by the immediate removal of the epoxy substrate by the extraction with *n*-pentane. The residual aqueous phase was poured onto an Amberlite XAD-2 column (1 × 30 cm for 10 ml of the phase). The column was washed with water (3 bed volumes) and eluted with 50% aqueous methanol (2 bed volumes). The residue obtained on the evaporation of the solvent from the eluate was dissolved in water containing *erythro*-1,2-dihydroxy-1-phenylpropane as an internal reference and subjected to h.p.l.c. carried out under the same conditions as mentioned above. In the chromatogram, two u.v.-absorbing peaks appeared at the same retention times as those of the synthetic conjugates **1** and **2**. They were separately eluted from the octadecylsilicone column and examined for the identity with authentic **1** and **2** by ultraviolet spectroscopy as well as by t.l.c. using a ninhydrin reagent. A quantitative study showed that the



S-(1-phenyl-2-hydroxyethyl) glutathione I



S-(2-phenyl-2-hydroxyethyl) glutathione 2

Fig. 1. Structural assignment of glutathione conjugates of phenyloxirane by proton n.m.r. spectroscopy. The spectra were recorded with resolved synthetic samples in D_2O containing 3-(trimethylsilyl)propionic acid-d_4 sodium salt as internal reference on a JEOL Model PS-100 100 MHz NMR spectrometer. * A double resonance technique was applied to assign these protons. The splitting patterns of the proton signal at 2.86 ppm was equivocal because it was partially overlapped with the doublet due to the methylene protons of the cysteine moiety. § The protons appeared as a signal (2H from integration) overlapped both with the singlet due to the methylene protons of the glycine moiety and with the triplet due to the methine proton of the glutamic acid moiety.

rate constant for the glutathione conjugation reaction under these biological conditions was 96.8 nmoles/mg protein/min and also that phenyloxirane was converted to the conjugates **1** and **2** in the ratio 3:2. Non-enzymatic glutathione conjugate formation occurred under these conditions, but it was less than five per cent of the biological reaction rate.

During the course of our investigation of a Raney nickel (W-1 grade in activity) treatment of the synthetic conjugates, which was attempted to give further support to the assigned structures of **1** and **2**, we found that this well known hydrogenolytic desulfuration method, in spite of its historically frequent use for the assignment of the structures of *S*-conjugates [13–16], was not applicable to the conjugates of epoxides. A gas-chromatography–mass spectrometric study of the Raney nickel reaction indicated that the expected desulfuration products, phenethyl alcohol specifically from **1** and methylphenylcarbinol also from **2** according to Pachecka *et al.* [11], were both yielded not only from **1** but from **2** alone (10 mM each) with the Ni reagent (1.16 mg/ml) in absolute ethanol under refluxing conditions. Moreover, the ratio of phenethyl alcohol to methylphenylcarbinol rapidly increased during the reaction within 60 min. At the last stage of the reaction, methylphenylcarbinol disappeared from the medium, but phenethyl alcohol remained. Besides both alcohols, phenyloxirane, ethylbenzene, and toluene were yielded from **1** as well as from **2** in the reaction. At the earlier stage of the reaction, the formation of phenyloxirane was significant, and it decreased rapidly. At the later stage of the reaction, increasing amounts of the hydrocarbons were detected. The hydrogenolysis reactions of the glutathione conjugates with Raney nickel are summarized as illustrated in Fig. 2 from the results of time course studies carried out by using phenyloxirane, phenethyl alcohol, methylphenylcarbinol as substrates. Phenethyl alcohol was the most stable among the oxygen-bearing phenylethanes, but it was also slowly deoxygenated to the hydrocarbons. From phenyloxirane were yielded all the detected hydrogenolysis products of the conjugates. Phenyloxirane was also yielded from methylphenylcarbinol by the catalytic action of Raney nickel but not from phenethyl alcohol. The carbinol was unstable to approximately the same extent as phenyloxirane under these conditions and yielded not only the hydrocarbons at higher rates but also phenethyl alcohol probably through the oxirane as intermediate.

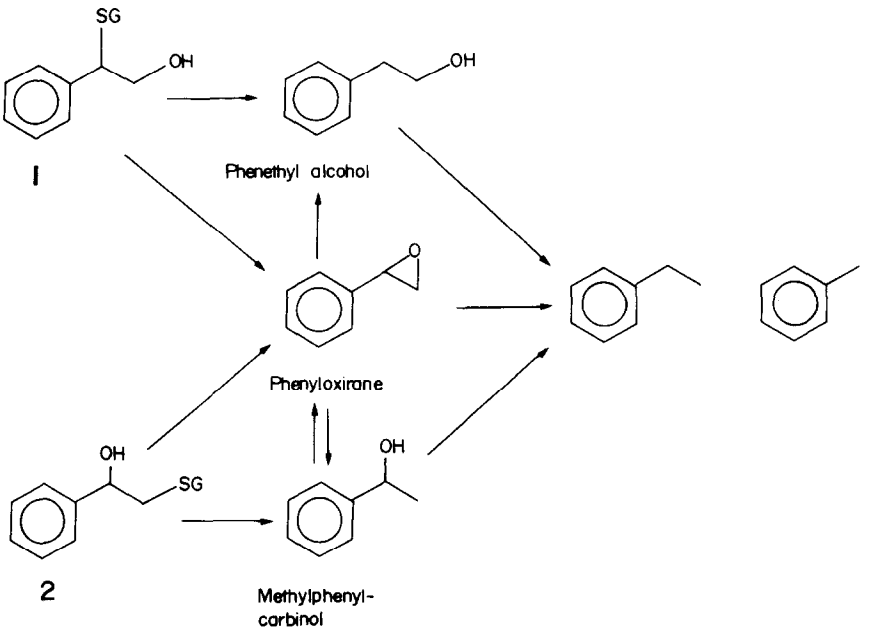


Fig. 2. Reactions of glutathione conjugates of phenyloxirane with Raney nickel in refluxing absolute alcohol. SG of the conjugates **1** and **2** represents the glutathione moiety. The products were all identified by gas-chromatography–mass spectrometry on a Shimadzu-LKB Model 9000 GC-MS; column—15% diethylene glycol succinate coated on Shimalite (60–80 mesh, 3 mm × 2 m), column temperature—50° on 120°, carrier gas—10 ml/min of helium, and ionization—20 eV. Under these conditions, retention times of the products were 2.8 and 5.0 min for toluene and ethylbenzene at 50° and 4.2, 10.7, and 16.7 min for phenyloxirane, methylphenylcarbinol, and phenethyl alcohol, respectively, at 120°. Intermediacy of phenyloxirane, phenethyl alcohol, and methylphenylcarbinol to toluene and ethylbenzene were confirmed by their reactions with Raney nickel under the same conditions as for the conjugates.

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