The Formation of Styrene Glutathione Adducts Catalyzed by Prostaglandin H Synthase

A POSSIBLE NEW MECHANISM FOR THE FORMATION OF GLUTATHIONE CONJUGATES*

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The metabolism of styrene by prostaglandin hydroperoxidase and horseradish peroxidase was examined. Ram seminal vesicle microsomes in the presence of arachidonic acid or hydrogen peroxide and glutathione converted styrene to glutathione adducts. Neither styrene 7,8-oxide nor styrene glycol was detected as a product in the incubation. Also, the addition of styrene 7,8-oxide and glutathione to ram seminal vesicle microsomes did not yield styrene glutathione adducts. The peroxidase-generated styrene glutathione adducts were isolated by high pressure liquid chromatography, and characterized by NMR and tandem mass spectrometry as a mixture of (2R)- and (2S)-S-(2-phenyl-2-hydroxyethyl)glutathione. (1R)- and (1S)-S-(1-phenyl-2-hydroxyethyl)glutathione were not formed by the peroxidase system. The addition of phenol or aminopyrine to incubations, which greatly enhances the oxidation of glutathione to a thyl radical by peroxidases, increased the formation of styrene glutathione adducts. We propose a new mechanism for the formation of glutathione adducts that is independent of epoxide formation but dependent on the initial oxidation of glutathione to a thyl radical by the peroxidase, and the subsequent reaction of the thyl radical with a suitable substrate, such as styrene.

Epoxidation is an important step in the enzymatic activation of many chemicals, including carcinogens, in biological systems. For example, certain dihydrodiol metabolites of polycyclic aromatic hydrocarbons are epoxidized to ultimate carcinogenic electrophiles, which react with DNA and form the biochemical lesion associated with the development of neoplasia. Epoxidation reactions are catalyzed by both the cytochrome P-450-containing monooxygenase system and by hydroperoxidase-dependent co-oxygenation catalyzed by prostaglandin H synthase. The mechanisms of these reactions are different: monooxygenases reduce molecular oxygen and transfer an oxygen atom directly to the substrate whereas peroxidases use peroxyl radicals as the oxidizing species. The peroxyl radicals are derived from unsaturated lipids present in the incubation mixture by prostaglandin H synthase (1) or are formed by hydroperoxidase-catalyzed oxidation of a reducing cofactor, such as phenylbutazone (2).

Epoxides are further metabolized by epoxide hydrolase to the corresponding diols, or they are conjugated with glutathione by the glutathione S-transferases. Styrene has been used as a model substrate for investigating the stereochemistry of P-450-dependent epoxidation (3, 4), and the subsequent hydrolysis (3, 5) and conjugation of its 7,8-oxide with glutathione (5, 6). Styrene is oxidized by the P-450 monooxygenase system to both (R)- and (S)-styrene 7,8-oxide which then react nonenzymatically or enzymatically with glutathione, resulting in the formation of four different S-substituted glutathione adducts (two pairs of diastereomers) as shown in Scheme 1. The two benzylic thioether products predominate in both enzymatic and nonenzymatic reactions (6, 7). Styrene is reportedly converted to styrene 7,8-oxide by soybean lipoxigenase in the presence of arachidonic acid (8) and by hemoglobin or myoglobin in the presence of H2O2 (9). These data suggest that styrene would also be oxidized to styrene 7,8-oxide by peroxyl radicals generated during prostaglandin biosynthesis. To investigate this possibility, we studied prostaglandin hydroperoxidase-dependent styrene metabolism. However, we did not observe the formation of styrene 7,8-oxide although we detected the formation of S-substituted styrene glutathione adducts when glutathione was present in the incubation mixtures. Our data suggest a new mechanism for the formation of glutathione adducts that is independent of epoxide formation but dependent on initial oxidation of glutathione to a thyl radical by the peroxidase and the subsequent reaction of this radical with a suitable substrate, such as styrene.

EXPERIMENTAL PROCEDURES

Materials
Arachidonic acid (99% pure) was obtained from Nu-Chek Prep, Inc., Elysian, MN. Aminopyrine, DETAPAC,1 glutathione, HEPES, horseradish peroxidase (Type VI), hydrogen peroxide (30%), indomethacin, and phenylbutazone were obtained from Sigma.
[8,14C]Styrene, 2.4 Ci/mol, and 97% radiochemically pure was obtained from Amersham Corp. [ring-2H]Styrene 7,8-oxide, 0.48 Ci/mol, 99% radiochemically pure, and [2,3H]glutathione, 5 Ci/mol, >98% pure, were purchased from New England Nuclear. Styrene, >99% pure, was obtained from Aldrich. Glacial acetic acid, ammonium hydroxide, and phenol were products of Mallinkrodt, Inc., Paris, NJ. HPLC grade methanol, HPLC grade water, and n-hexane, were purchased from Fisher. Anhydrous analytical grade glycerol and potassium phosphate monobasic were obtained from J. T. Baker.

1 The abbreviations used are: DETAPAC, diethylenetriaminepentacetate acid; FAB, fast atom bombardment; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HPLC, high pressure liquid chromatography; MS, mass spectrometry; RSV, ram seminal vesicles; MS-MS, tandem mass spectrometry.

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**Peroxidase-catalyzed Formation of Glutathione Conjugates**

Chemical Co. Hydrofluor was purchased from National Diagnostics, Somerville, NJ, and Aquasol from New England Nuclear.

**Methods**

**RSV Microsomal Preparation**—Ram seminal vesicles were obtained from a slaughterhouse and stored at −70 °C until used. Tissue was thawed and trimmed of excess fat and connective tissue, and microsomes were prepared as previously described (10) with the exception that homogenization was carried out in 1.15% KCl containing 10 mM HEPES, pH 7.5, and 1% bovine serum albumin. Microsomes were resuspended in 1.15% KCl containing 10 mM HEPES, pH 7.5. Protein determination was made using the method of Lowry et al. (11).

Microsomal prostaglandin H synthase activity was determined prior to each experiment by measuring oxygen incorporation into arachidonic acid with a Clark-type oxygen electrode.

**Incubation Conditions**—Incubation mixtures consisted of 100 mM HEPES buffer, pH 7.6, with 1 mM DMAPC, 100 mM [14C]stilbene (0.5 μCi), 1 mM glutathione, and 1 mg of RSV microsomal protein in a final volume of 1 ml. In some experiments [3H]glutathione (5 μCi) was used. When H2O2 replaced arachidonic acid it was present at 100 μM and when horseradish peroxidase was used as the peroxidase it was present at a concentration of 20 μg/ml. The reaction mixtures were preincubated at 37 °C for 2 min in the absence of either arachidonic acid or H2O2. The peroxidase substrate was then added to initiate the reaction, followed by incubation for 5 min. The reaction was stopped by the addition of 1 ml of cold methanol. Incubation mixtures designed to test for the formation of styrene 7,8-oxide at high styrene concentrations consisted of 100 mM potassium phosphate, pH 7.6, 40 mM [3H]styrene, 5 mM H2O2, and 200 μg of horseradish peroxidase in a final volume of 2 ml. This reaction was studied alone and in the presence of either or both 5 mM glutathione and 500 μM phenol. The reactions were stopped by immersion in an ethanol/dry ice bath and samples analyzed by HPLC without further treatment.

**Preparation of Samples for Analysis**—Incubation mixtures containing methanol were centrifuged to remove precipitated protein and then extracted with 4 ml of hexane. The hexane extraction was repeated twice to remove residual styrene and any styrene 7,8-oxide present. The water/methanol phase was then evaporated to dryness under reduced pressure in a Speed Vac concentrator (Savant). The residual solid was dissolved in 0.25 ml of HPLC grade water prior to analysis.

**HPLC Analysis**—A Waters HPLC system consisting of a model U6K injector, two model 600A solvent delivery pumps, a model 720 system controller, and a 2 module radial compression unit containing a Waters radial pak 5-μ C8 cartridge of 8 mm inner diameter was used for HPLC analysis. A Flo-one radioactive flow detector (Radioimmuno Instruments and Chemical Co., Inc., Tampa, FL) linked to a Qume visual display and a C-R plotter 8510 was used to measure and record the radioactivity in the HPLC eluant, at 6-s intervals. Hydrofluor was used as the scintillation fluid. Radioactivity was corrected for quenching and converted to dpm/fraction.

The elution system consisted of a 10-min isocratic water phase followed by a linear gradient from 0 to 100% methanol over the next 30 min. This was followed by the reverse of this gradient from 100% methanol to 100% water over the next 10 min. The flow rate was 2 ml/min. This effectively separated the styrene glutathione adducts from styrene and other potential metabolites such as styrene 7,8-oxide and styrene glycol (Fig. 1).

**HPLC Resolution of Styrene 7,8-Oxide Glutathione Diastereomers**—The HPLC system described above was used, and the conditions were those initially described by Hernandez et al. (7). A stock solution of Tris-phosphate (pH 7.0) was prepared as follows: 5.1 ml of 85% phosphoric acid were added to 1000 ml of HPLC grade water and neutralized to pH 7.0 with Tris-base.

Buffer A consisted of 500 ml of Tris-phosphate, pH 7.0, plus 500 ml of a 50 mM sodium sulfate solution. Buffer B contained 475 ml of stock solution, 25 ml of methanol, and 500 ml of 50 mM sodium sulfate.

For separation the sample was injected in buffer A which was pumped through the column at 2 ml/min for 30 s. Elution was then continued with buffer B for 60 min, during which time 0.8-ml fractions were collected. The eluted fractions were each added to 5 ml of Aqusol, and their radioactive content was determined in a Packard 460C liquid scintillation spectrometer.

**Preparation of Styrene Glutathione Adducts for Structural Identification**—The incubation mixtures contained 1 mM [14C]stilbene, 5 mM glutathione, 1 mM H2O2, 500 μM phenol, and 5 mg of horseradish peroxidase in 10 mM potassium phosphate buffer, pH 7.6 (200 ml).

After incubation at 37 °C for 10 min the reaction mixture was extracted with hexane (3 × 800 ml) and the aqeous phase evaporated to dryness under reduced pressure. The residual solid was dissolved in 1 ml of water and 100-μl quantities of this solution were added to 10 individual Sep-Pak cartridges (Waters) which had been sequentially washed with 10 ml of methanol, 10 ml of water, and 10 ml of 0.1 M acetic acid. Each Sep-Pak was then washed with 6 ml of 0.1 M acetic acid and 6 ml of water. This procedure was shown to remove most of the excess glutathione and phosphate buffer. The styrene glutathione adducts were finally eluted with 2.5 ml of methanol. This solution was filtered through a 0.45-μm fluoropolymer membrane (Acro LC13-Gelman) and then evaporated to dryness.

The 10 fractions from the parent incubation were then eluted individually from a Sep-Pak G2 preparative column, 7.8 mm × 50 cm (Waters), with an isotropic 0.1 M ammonium acetate solution (pH 5.35) for 10 min followed by a linear ammonium acetate/methanol gradient going from 0 to 100% methanol over the next 30 min. For the next 10 min, the gradient was reversed from 100% methanol to 100% 0.1M ammonium acetate. Some (10%) of the eluent was channeled into the radioactive detector to measure radioactivity in each fraction while the remaining 90% of each fraction was collected in vials at 30-s intervals. Those vials which corresponded to the major radioactive peak were pooled and evaporated to dryness for MS and NMR analysis.

**Mass Spectral Analysis by Fast Atom Bombardment**—The sample was dissolved in methanol and placed onto a FAB target, methanol was allowed to evaporate, and the sample was then covered with a glycerol-oxalic acid matrix. The oxalic acid was included to enhance the formation of the protonated molecular ion. Analyses were performed on a VG ZAB 4F tandem instrument of BE-EB geometry (VG Analytical, Manchester, England). The xenon beam generated under FAB conditions gave a collision energy of 8 keV. The secondary ions were accelerated at 8 keV, and the spectrum was recorded on a DATA System PDP 11/250 (VG Analytical). A MS-MS spectrum was measured by selecting the m/z 428 ion with MS1 (BE).

**Nuclear Magnetic Resonance**—The natural abundance, proton-decoupled 13C NMR spectrum was obtained in the Fourier transform mode on a GE QE300 spectrometer. Parameters used in acquiring the NMR spectrum were as follows: spectral width 10,000 Hz; pulse angle 35°; repetition time between pulses 2 s; data points 16K; exponential broadening 1.0 Hz; and probe temperature 30 °C. A small amount of p-dioxane was added as internal reference and chemical shifts were converted to the tetramethylsilane (TMS) scale using the relationship "TMS = dioxane + 66.5."

**RESULTS**

**Formation of Styrene Glutathione Adducts**—An HPLC system was developed which separates styrene, styrene 7,8-oxide, styrene glycol, and the styrene 7,8-oxide glutathione adducts. In this system the thioether conjugates were not resolved into individual diastereomers. Analysis of incubation mixtures containing [14C]styrene, RSV microsomes, and arachidonic acid showed little, if any, metabolism of styrene. The addition of glutathione to the incubation mixture resulted in the for-
formation of metabolites which co-eluted with authentic styrene 7,8-oxide glutathione adducts. However, the formation of styrene 7,8-oxide and styrene glycol was not detected under these conditions (Fig. 1).

Confirmation that this metabolite contained both styrene and glutathione was obtained by incubation of [14C]styrene with [3H]glutathione in the presence of arachidonic acid and ram seminal vesicle microsomes. After hexane extraction of the incubation mixture to remove unmetabolized styrene and filtration to remove precipitated protein, the resultant aqueous phase was evaporated and chromatographed on a Waters C18 radial pak cartridge, with the HPLC system that separates the styrene 7,8-oxide glutathione adducts into four peaks, each containing a single diastereomer (7).

As seen in Fig. 2 the incubation mixture which contained both [14C]styrene and [3H]glutathione was resolved into two quantitatively equivalent peaks which eluted at 50 to 55 min and which contained both 3H and 14C. The large 3H peak at the origin was unchanged glutathione while the smaller 3H peak at 20 min co-eluted with glutathione disulfide. These results confirm that adducts containing equimolar concentrations of styrene and glutathione are formed by prostaglandin H synthase.

The formation of these adducts by RSV microsomes was dependent on arachidonic acid. Moreover, addition of the prostaglandin H synthase inhibitor, indomethacin, significantly depressed the formation of these compounds. Adduct formation was also dependent on the presence of glutathione in the incubation mixture, and little or no adduct was formed with heat-denatured microsomes (Table I). The use of H2O2 in place of arachidonic acid also resulted in formation of styrene glutathione adducts and a qualitatively similar pattern of metabolites was observed; no styrene 7,8-oxide or styrene glycol formation was detected. Little or no conjugate formation was observed in the absence of H2O2 or of glutathione or with heat-denatured microsomes (Table II).

The addition of [14C]styrene 7,8-oxide to incubation mixtures containing RSV microsomes and glutathione did not result in the formation of styrene glutathione adducts. However, styrene 7,8-oxide was extensively hydrolyzed to styrene glycol in accordance with the epoxide hydrolase activity present in these microsomes (12). Incubations containing arachidonic acid or H2O2 and styrene 7,8-oxide also did not support adduct formation. Styrene 7,8-oxide was recovered essentially unchanged from incubation mixtures of [14C]styrene oxide,
horseradish peroxidase, glutathione, and H₂O₂, although a small amount of styrene glycol and styrene 7,8-oxide glutathione adducts formed nonenzymatically.

Previously, we showed that the addition of phenylbutazone to an incubation mixture of H₂O₂ and RSV microsomes greatly enhanced the metabolism of 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene to its anti-7,8-dihydrodiol-9,10-epoxide derivative (2). The addition of phenylbutazone to incubation mixtures containing styrene, RSV microsomes, glutathione, and H₂O₂ did not result in detectable formation of styrene 7,8-oxide or styrene glycol, and no additional styrene metabolites were detected either in the presence or absence of glutathione (data not shown). These results support the conclusion that the hydroperoxidase activity of RSV catalyzes the formation of styrene glutathione adducts in the absence of styrene 7,8-oxide formation.

The formation of styrene glutathione adducts was also catalyzed by horseradish peroxidase (Table III). HPLC analysis of these incubation mixtures also demonstrated adduct formation with no apparent formation of styrene 7,8-oxide or styrene glycol. Once again, adduct formation was dependent on presence of enzyme, substrate (H₂O₂), and glutathione in the incubation mixture. Significantly greater amounts of the styrene glutathione conjugates were formed by horseradish peroxidase than by prostaglandin H synthase in accordance with the greater activity of horseradish peroxidase. The products obtained with horseradish peroxidase were shown to coinocubate with the adducts from RSV microsomal incubations while the direct addition of [¹⁴C]styrene 7,8-oxide to incubations containing horseradish peroxidase, H₂O₂, and glutathione resulted in essentially quantitative recovery of unchanged styrene 7,8-oxide.

Recently, we showed that glutathione is oxidized by prostaglandin H synthase and horseradish peroxidase to a thyl radical (13, 17). Although glutathione is not a good substrate for the peroxidases, the addition of either phenol or aminopyrine to the incubation mixture greatly increased thyl radical formation (14, 23). The addition of aminopyrine or phenol to incubations containing styrene, glutathione, and peroxidase greatly increased styrene glutathione adduct formation (Tables I–III). An approximate 10-fold increase in adduct formation was observed with phenol and RSV in the presence of arachidonic acid (Table I) or H₂O₂ (Table II) and with the horseradish peroxidase/H₂O₂ system (Table III).

**Structural Identification of Styrene Glutathione Adducts**

Large scale incubation mixtures containing the horseradish peroxidase/H₂O₂ system and phenol were used to prepare sufficient quantities of material for structural identification.

### Table III

**Formation of styrene glutathione adducts by horseradish peroxidase**

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Styrene glutathione adducts</th>
<th>nmol/incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system*</td>
<td>12.8 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>− H₂O₂</td>
<td>0.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>− Glutathione</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>− Horseradish peroxidase</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>+ Phenylbutazone*</td>
<td>15.3 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>+ Aminopyrine*</td>
<td>29.5 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>+ Phenol*</td>
<td>83.0 ± 12.7</td>
<td></td>
</tr>
</tbody>
</table>

* The complete system contained 100 mM HEPES, pH 7.6, 1 mM DETAPAC, 100 µM [¹⁴C]styrene, 1 mM glutathione, 100 µM H₂O₂, and 20 µg of horseradish peroxidase in a final volume of 1 ml.

**Fig. 3.** The HPLC profile of radioactive products generated during the preparation of [¹⁴C]styrene glutathione adducts for structural identification (see "Methods"). The products were extracted with hexane and injected onto a C十八µBondapak steel column (7.8 mm × 30 cm) and eluted with the 0.01 M ammonium acetate (pH 5.35)/methanol system as described under "Methods." The elution profile showed four peaks. The major product (peak 1) had a retention time of 27.3 min while the minor products 2, 3, and 4 had retention times of 30.4, 31.5, and 33.8 min, respectively.

**Fig. 4.** HPLC profiles of the styrene 7,8-oxide glutathione adducts formed from [¹⁴C]styrene 7,8-oxide and glutathione by rat hepatic cytosol (—) and the products of incubation of [¹⁴C]styrene, glutathione, and arachidonic acid with prostaglandin H synthase (—). The products were extracted with hexane and then injected onto a 5-µm C十八 radial pak column housed in a Z-compression module and chromatographed as described under "Methods."
glutathione, catalyzed by prostaglandin hydroperoxidase or horseradish peroxidase, does not proceed via an epoxide intermediate. We were unable to detect styrene 7,8-oxide or styrene glycol in our peroxidase-containing incubation mixtures under any conditions. Moreover, the addition of styrene 7,8-oxide to RSV microsomal incubations containing glutathione, catalyzed by prostaglandin hydroperoxidase or horseradish peroxidase, does not proceed via an epoxide intermediate, and the absence of arachidonic acid, did not yield enzymatically formed styrene oxide glutathione adducts. However, styrene glycol and unreacted styrene oxide were present in these reaction mixtures, which is in agreement with the presence of epoxide hydrolase (13) in RSV. Cytochrome P-450 monooxygenase activity is also absent from RSV microsomes (18).

The oxidation of styrene to styrene 7,8-oxide by P-450 monoxygenases followed by glutathione S-transferase-catalyzed conjugation results in the formation of two pairs of diastereomeric conjugates (Scheme 1) (6). Thioether adducts are formed by thiol (of glutathione) reaction with both the benzylic oxirane carbon atom (C-7) and the terminal oxirane carbon (C-8) of styrene 7,8-oxide. Nonenzymatically, glutathione reacts preferentially with the benzylic position, with a ratio for reaction at C-7/C-8 of approximately 2:3. With peroxidases, reaction occurs only with the terminal carbon atom (C-8) of styrene, and the two possible diastereomers are formed in equal amounts.

Previously, we showed that glutathione is oxidized to a thyl radical by prostaglandin hydroperoxidase (13) or horseradish peroxidase (17). Furthermore, the addition of a good reducing cofactor for the peroxidase greatly enhances the formation of the thyl radical. The reducing cofactor is initially oxidized by the peroxidase to a free radical which is subsequently reduced by glutathione with the resultant formation of a thyl radical (14). Formation of styrene glutathione adducts was greatly enhanced by the presence of a reducing cofactor (phenol or aminopyrine) (14) in the reaction mixture. These findings, in addition to the specific addition of glutathione to the terminal carbon atom (C-8) of styrene and the absence of an epoxide (styrene 7,8-oxide) intermediate, suggest a new mechanism for the formation of glutathione adducts of xenobiotics.

**DISCUSSION**

The formation of styrene glutathione adducts occurred during prostaglandin biosynthesis in RSV microsomes. The reaction was dependent on the presence of the prostaglandin H synthase substrate, arachidonic acid, and was inhibited by indomethacin. This indicates that styrene glutathione adduct formation is catalyzed by prostaglandin H synthase. Substitution of H2O2 for arachidonic acid also supported conjugate formation which strongly suggested that prostaglandin hydroperoxidase was responsible for styrene metabolism. Thioether formation was also observed in incubations containing horseradish peroxidase, H2O2, styrene, and glutathione. The chemical identity of the diastereomeric styrene glutathione adducts formed was (2R)- and (2S)-S-(2-phenyl-2-hydroxyethyl)glutathione, as determined by HPLC, FAB MS-MS, and NMR spectroscopic analyses.

The formation of the thioether adducts from styrene and glutathione, in the presence of rat cytosolic glutathione S-transferases (6, 7) were clearly separated. However, only two adducts were formed from styrene and glutathione by the peroxidase system. These products co-eluted with (2S)-S-(2-phenyl-2-hydroxyethyl)glutathione and (2R)-S-(2-phenyl-2-hydroxyethyl)glutathione, respectively. Thus, (1R)- and (1S)-S-(1-phenyl-2-hydroxyethyl)glutathione were apparently not formed by the peroxidase-dependent reaction.

The chemical identity of the peroxidase-generated styrene glutathione adducts was established by MS and NMR analyses. Fig. 5 shows the mass spectrum of the metabolite in peak 1 (Fig. 3) prepared from large scale incubation mixtures and analyzed by FAB using MS-MS techniques. The spectrum shown in Fig. 5 is consistent with the structure S-(2-phenyl-2-hydroxyethyl)glutathione and was identical to that obtained from authentic synthetic standards. The major ion observed was 428, corresponding to the MH+ ion. Another major ion was 410, representing the loss of H2O from the MH+ ion. m/z 353 and 299 are analogous to m/z 337 and 283, respectively, in the spectrum of the nonhydroxylated compound. m/z 335 and 281 correspond to loss of H2O from the 353 and 299 ions. Fig. 6 is the 13C NMR spectrum of the metabolite in peak 1 (Fig. 3). The peak at 66.49 ppm is an internal reference. The NMR spectrum contained 16 resonances which are in agreement with the resonances reported earlier for individual styrene 7,8-oxide glutathione adduct diastereomers by Yagen et al. (15).

![Fig. 5. Mass spectrum of peroxidase-produced (2R,2S)-S-(2-phenyl-2-hydroxyethyl)glutathione. Spectrum was obtained using FAB and MS-MS techniques described under "Methods."](image-url)

![Fig. 6. 13C NMR spectrum of peroxidase-produced (2R,2S)-S-(2-phenyl-2-hydroxyethyl)glutathione.](image-url)
We propose a free radical mechanism for the formation of diastereomeric S-(2-phenyl-2-hydroxyethyl)glutathione as illustrated in Scheme 2.

\[ RS^* + \overset{\text{O}}{\overset{\text{CH-CH}_2}{\text{CH=CH}_2}} \rightarrow \overset{\text{CH-CH}_2^{-}}{\overset{\text{S-R}}{\text{S}}} \]

\[ \overset{\text{O}_2}{\overset{\text{OO}^*}{\text{S}}} \]

**SCHEME 2**

A glutathione thiol radical, formed directly by the peroxidase or indirectly via the oxidation and reduction of a reducing cofactor, adds to the alkyl double bond of styrene. A carbon-centered radical results which reacts with molecular oxygen to form a peroxyl radical. The peroxyl radical eventually is reduced to the corresponding alcohol.

The addition of thyl radicals to olefinic double bonds is well established, and a variety of stable products are isolated, depending on the reaction conditions (18, 19). The addition of thiyl radicals to \( \alpha \)-methylstyrene (20, 21) or indene (22) is known to occur exclusively at the terminal carbon of the double bond. The subsequent addition of oxygen to the intermediate carbon-centered radical eventually produces the hydroxyl group at the adjacent carbon. Thus, the stereochemistry we observed for peroxidase-catalyzed formation of styrene glutathione adducts is identical to that reported for thyl radical addition to olefins.

In summary, we propose that two possible mechanisms exist for the observed formation of glutathione conjugates or adducts from styrene. The first is the accepted classical reaction sequence in which styrene is oxidized by P-450 monooxygenases to its 7,8-epoxide. The epoxide then reacts enzymatically or nonenzymatically with glutathione to yield S-substituted adducts (Fig. 7). A second mechanism for the formation of glutathione adducts of olefins is free radical in nature; it involves the addition of a peroxidase-generated thyl radical to a double bond. Further studies to identify the nature of the free radical intermediates in prostaglandin hydroperoxide-dependent formation of S-(2-phenyl-2-hydroxyethyl)glutathione are required.

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