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Hepatic microsomal oxidation of styrene to 4-hydroxystyrene 7,8-glycol via 4-hydroxystyrene and its 7,8-oxide as short-lived intermediates

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4-Hydroxystyrene **3** is a urinary metabolite of the plastic monomer, styrene, in the human [1] as well as in the rat [2–4] and has recently been demonstrated to be a sole specific rearrangement product of the highly reactive and mutagenic epoxide, styrene, 3,4-oxide (1-vinylbenzene 3,4-oxide) **2**, which has a half-life of 4.3 sec at 37° and pH 7.4 in an aqueous solution, and has been considered as a putative intermediate in the biotransformation of **1** to **3** [5, 6] (Fig. 1). However, nothing has been reported on the hepatic formation of **3** from **1** *in vitro*. In the present communication, we wish to report that the radioactive metabolite **3** formed from ^{14}C-labelled **1** is undetectable in the hepatic microsomal incubation system fortified with NADPH without using a large amount of unlabelled **3** as a trapping agent for the metabolite, because it is rapidly oxidized by monooxygenase to yield 4-hydroxystyrene 7,8-glycol (4′-hydroxyphenylethane-1,2-diol) **5** via the highly reactive intermediate, 4-hydroxystyrene 7,8-oxide (4′-hydroxyphenyloxiran) **4**.

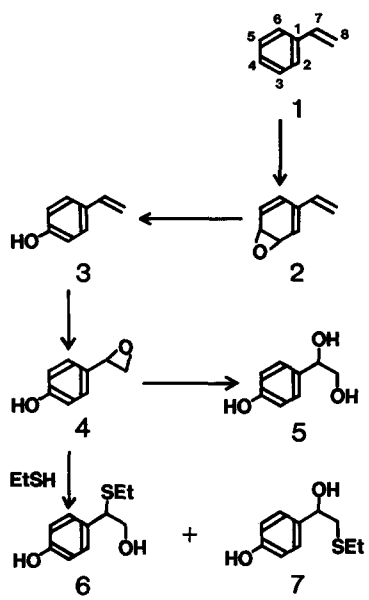


Fig. 1. Oxidation of styrene to 4-hydroxystyrene and 4-hydroxystyrene 7,8-glycol via highly reactive epoxides. **1**: Styrene, **2**: styrene 3,4-oxide, **3**: 4-hydroxystyrene, **4**: 4-hydroxystyrene 7,8-oxide, **5**: 4-hydroxystyrene 7,8-glycol, **6**: 2-(4′-hydroxyphenyl)-2-ethylmercaptoethanol, **7**: 1-(4′-hydroxyphenyl)-2-ethylmercaptoethanol.

Styrene **1** (0.1 mM), redistilled before use and dissolved in acetone (1.2% v/v), was aerobically incubated at 37° for 30 min in a final volume of 5 ml of 0.1 M phosphate buffer, pH 7.4, with liver microsomes (25 mg protein/ml) from male Wistar rats (100–120 g), pretreated with phenobarbital as previously reported [7] in the presence of an NADPH-generating system consisting of NADP (1 mM), glucose 6-phosphate (10 mM), glucose 6-phosphate dehydrogenase (1 IU/ml) and magnesium chloride (5 mM) in an air-tight flask.

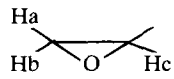
The mixture was extracted with an equal volume of ethyl acetate in the presence of a saturating amount of sodium chloride. From the organic phase separated by centrifugation, the solvent was evaporated through a fractional condenser to obtain a condensate, with minimal loss of the volatile metabolites. A g.l.c.–m.s. study showed the condensate not to contain any detectable amount of the phenol **3**. However, it contained the triol **5** which had a retention time of 6 min as a tri-TMS ether on a 1.5% OV-101 column (coated on 80–100 mesh Chromosorb W, 2 m × 3 mm) eluted at 200° with 40 ml He/min; chemical-ionization m.s., recorded with isobutane as a reagent gas at 0.5–1 Torr, *m/z* (relative intensity, %) at an ionization voltage of 100 eV with 100 A ionization current at an ion-source temperature of 250°: 371 ($M^+ + 1$, 18), 355 (25), 282 (40), 269 (15), 268 (30), 267 (100) and 147 (20). The mass spectrum and the retention time were identical with those of the corresponding authentic specimen synthesized as previously reported [8].

[7-^{14}C] **1** [9] (4 μCi/μmole, 0.1 mM) was incubated in the presence of unlabelled **3** (0.8 mM) in a final volume of 250 ml of the mixture under the aforementioned conditions in order to trap radioactive **3** formed during incubation. Using the liquid scintillation counting-absorptiometry-method, **3** isolated as a phenolic fraction from the incubation mixture was found to contain 243 pCi/μmole of radioactivity at 260 nm after it was purified to the constant radioactivity to absorbance ratio by twice successive use of an octadecylsilicone column (ODS, Nucleosil 7C_{18}, 5 μ in particle size, 30 cm × 4 mm) and a silica column (Spherisorb silica, 5 μ, 30 cm × 4 mm) eluted at 1 ml/min by MeOH–H_2O (1:1) and by *n*-hexane-*iso*-PrOH (200:1), respectively. Under the h.p.l.c. conditions used, **3** was eluted at 11.6 min together with its isomers, 2- and 3-hydroxystyrenes [10], as an inseparable peak from the ODS column as far as the authentic phenols were co-chromatographed. The phenol **3**, however, was separated from the eluted phenolic fraction by subsequent h.p.l.c. on the silica column; retention times of 2-hydroxystyrene, 3-hydroxystyrene and **3** were 24, 33 and 38 min respectively. No detectable amount of radioactivity was incorporated into the phenolic fraction when boiled microsomes were used.

The fact that the stable metabolite **3** yielded from **1** could not be detected from the incubation mixture without using the isotope-trapping method led us to assume that **3** would have been a very good substrate for rat liver microsomal P-450 by which its vinyl group could be rapidly oxidized. Actually, **3** (2 mM) disappeared from the incubation mixture at a rate of 2.8 times faster (8.2 nmol/mg microsomal protein/min) than the vinyl group of **1** (2 mM) was oxidized under the same conditions. It should be noticed that **1** has been recognized as one of the most oxidizable substrates in rat liver microsomes and yields styrene 7,8-glycol (phenylethane-1,2-diol) as the major metabolite via styrene 7, 8-oxide (phenyloxiran) by the sequential action of P-450 and epoxide hydrolase [11–16].

Addition of TCPO (3,3,3-trichloropropene 1,2-oxide, 1 mM), a potent microsomal xenobiotic epoxide hydrolase inhibitor, to the incubation mixture did not affect the rate of formation of the triol **5** from **3**, although under the same conditions TCPO strongly inhibited (about 90% inhibition) the formation of styrene 7,8-glycol from **1** and, instead, accumulated unhydrolysed styrene 7,8-oxide in the mixture as had been demonstrated [15].

A synthetic approach to the problem on obligatory intermediacy of 4-hydroxy-styrene 7,8-oxide (4′-hydroxyphenyloxiran) **4** in the microsomal transformation of **3** to **5** demonstrated that **4** was found to be too reactive to be isolated from the aqueous medium because of its facile conversion to **5** in water. Although an olefin oxide, in general, is more stable at alkaline pH than at pH 7.4 [17], the stable derivative of the epoxide, **4** benzoate (m.p. 109–110°), yielded **5** as the major product without concomitant formation of less polar **4** when treated at room temperature with an equimolar ratio of sodium hydroxide in aqueous methanol, monitoring the time course of the reaction by h.p.l.c. The unstable epoxide **4** behaved as a potent electrophile, so that it might react with acetone to form an acetonide of **5** when the benzoate of **4** was treated with a slight excess of sodium hydroxide in acetone at room temperature. The benzoate was synthesized from **3** benzoate by the reaction with *m*-chloroperbenzoic acid in chloroform; m.s. *m/z*: 240 (M^+); ^{1}H n.m.r. $\delta_{TMS}^{CDCl_3}$ ppm: 2.75 (Ha, *dd*), 3.13 (Hb, *dd*), 3.86 (Hc, *dd*), and *J*ab = 5.2 Hz, *J*ac = 3 Hz, *J*bc = 4.5 Hz for



u.v. λ_{max}^{EtOH} nm (log ε): 230 (4.29); i.r. ν_{max}^{KBr} cm^{-1}: 1735 ($\nu_{c=0}$).

However, indirect evidence was obtained for the microsomal formation of the unstable epoxide **4** as an obligatory intermediate from **3** to **5** by incubating **3** (2 mM) in the presence of the nucleophile, ethyl mercaptan (10 mM), under the aforementioned conditions. From the incubation mixture were isolated, together with **5**, two isomeric α-hydroxyethyl sulphides **6** and **7** in the ratio 1.5:1 which were identified by g.l.c.–m.s. after being methylated with diazomethane (Fig. 2). The derivatized sulphides were obtained as a mixture by the reaction of 4-methoxystyrene 7,8-oxide [18] with ethyl mercaptan in an aqueous sodium hydroxide solution and identified with **6** and **7** by g.l.c.–m.s.

A stoichiometric study of the microsomal oxidation of **3** to **5** in the absence of ethyl mercaptan showed the presence of a large discrepancy between **3** consumed and **5** formed; the latter was only 60% of the total consumption of the former. Under the conditions used, **5** added to the microsomal incubation system was recovered almost quantitatively, indicating the triol to be a poor substrate for microsomal monooxygenase. The observed discrepancy may suggest that **4** formed from **3** would covalently bind as an electrophile because of its high reactivity with microsomal proteins in a manner similar to the reaction with ethyl mercaptan.

Carbon monoxide strongly inhibited the microsomal oxidation of **3** to **5**, but SKF 525-A, metyrapone, and 7,8-benzoflavone all showed little inhibitory effect on the reaction at 0.1 mM each and only a little effect even at 1 mM. Pretreatment of the animals with phenobarbital induced the rates of formation of **5** from **1** 4.4 times and from **3** 1.6 times as high as those in the untreated animals.

The triol **5** might be obtained from styrene 7,8-glycol via its arene oxide as a putative intermediate which has been

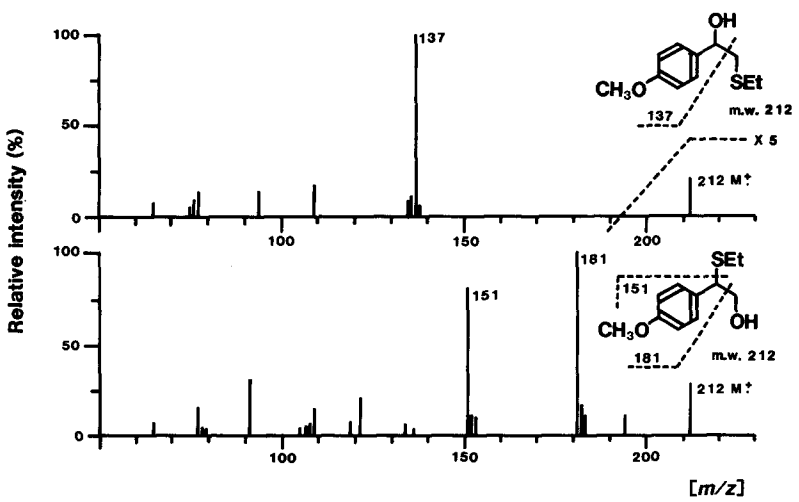


Fig. 2. Mass spectra of ethyl sulphides formed from 4-hydroxystyrene via the possible intermediate, 4-hydroxystyrene 7,8-oxide, in rat liver microsomes. The sulphides were methylated with diazomethane in ether following extraction from the incubation mixture. G.l.c.–m.s. conditions—column: 1.5% OV-17 (Chromosorb W, 80–100 mesh, 3 mm × 2 m); column temperature: 165°; carrier gas: 40 ml He/min; ionization voltage: 70 eV. Retention times: 12.8 min (upper) and 10.8 min (bottom).

suggested to play a role in covalent binding of the glycol to hepatic microsomal protein [19]. In the present investigation, however, **5** was not detected in the microsomal system containing styrene 7,8-glycol as a substrate by g.l.c.–m.s. carried out under the same conditions as those for detecting **5** from **1**.

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