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THE METABOLISM OF STYRENE OXIDE IN THE ISOLATED PERFUSED RAT LIVER

Identification and Quantitation of Major Metabolites

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ABSTRACT

Isolated perfused rat livers rapidly metabolized ^{14}C-styrene oxide. A large proportion of the administered radioactivity was excreted in the bile as a single compound that was identified as S-(1-phenyl-2-hydroxyethyl)glutathione by comparison with an authentic synthetic standard. The circulating perfusate was found to contain approximately equal amounts of styrene glycol, mandelic acid, and the glutathione derivative.

Styrene, with an annual production of about 2,000,000 tons in the United States (1), is a widely used industrial chemical. As such, it possesses potential as an environmental pollutant. Metabolic studies (2, 3) have demonstrated that styrene is oxidized *in vivo* at the vinylic double bond to give a variety of urinary metabolites. Among these are hippuric acid, styrene glycol monoglucuronide, phenylglyoxylic, and mandelic acids. *In vitro* studies (4) showed that rat liver microsomal fraction converts styrene to styrene glycol. These data indicate that the primary oxidative metabolite of styrene is styrene oxide, which then undergoes further biotransformation (5).

Styrene oxide is an epoxide and is potentially an alkylating agent. Alkene and arene oxides have been suggested to be ultimate toxicants which covalently bind to cellular macromolecules, leading to carcinogenesis, mutagenesis, and cytotoxicity (6–8). Recently, styrene oxide has been found to be mutagenic to bacteria (9), which emphasizes the need for a thorough understanding of the metabolism of this chemical.

Alkene and arene oxides are converted to the corresponding diols and dihydrodiols by rat liver microsomal epoxide hydrase (for reviews, see 6, 7). The soluble glutathione S-transferases of rat liver catalyze the reaction of glutathione with both alkene and arene oxides. Styrene oxide has been shown to be a substrate for both pathways (10). Inasmuch as these enzymes have different subcellular locations in the hepatic parenchymal cell, it is difficult to assess their relative importance by *in vitro* studies. In the rat, both specific and total activities of hepatic glutathione S-transferase greatly exceed those of microsomal epoxide hydrase when styrene oxide is used as substrate (10). We have therefore studied the metabolism of styrene oxide in the isolated perfused rat liver, a preparation with intact cellular structure, with the purpose of determining the relative quantitative importance of the hydration and glutathione-conjugation pathways for this substrate. The hepatic preparation was utilized because liver is normally the major site for oxidative xenobiotic metabolism in mammals and because bile could be readily collected. This was important because biliary excretion was expected to be a major route for excretion of the glutathione conjugate. In intact animals, gut metabolism may obscure the nature of the biliary biotransformation products, and there may also be substantial fecal excretion of these metabolites.

Materials and Methods

[8-^{14}C]Styrene oxide was purchased from New England Nuclear (Boston, Mass.); specific activity, 0.37 mCi/mmol. The radiochemical purity was 98.5%, as demonstrated by TLC[2] analysis in hexane. It was diluted as necessary with unlabeled styrene oxide (East-

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[2] Abbreviations used are: TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; UV, ultraviolet.

man Organic Chemicals, Rochester, N.Y.). Styrene glycol and mandelic acid were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Glutathione was purchased from Sigma Chemical Co. (St. Louis, Mo.).

S-(1-Phenyl-2-hydroxyethyl)glutathione was prepared as follows: Glutathione (614 mg, 2 mmol) was dissolved in water (4 ml) containing NaOH (160 mg, 4 mmol). Styrene oxide (240 mg, 2 mmol) was added, followed by sufficient ethanol to make the mixture homogeneous. After 3 hr the mixture was acidified to pH 3.5 with concentrated HCl, diluted with water (25 ml), and extracted three times with 10-ml portions of ethyl acetate. The aqueous residue was stirred with Amberlite XAD-2 resin (Applied Science Laboratories, State College, Pa.). The resin (5 g) was collected by filtration, washed well with water, and then four times with 25-ml portions of methanol. The methanolic washings were evaporated under reduced pressure to yield a clear gum. This was triturated with acetone to give a white solid, which was collected and crystallized from water/acetone, m.p. 172–173°. It was homogeneous by TLC analysis (see below) and gave a purple color with ninhydrin.

Anal: Found, C, 50.4; H, 6.0; N, 9.7; S, 7.4%. $C_{18}H_{25}O_7N_3S$ requires C, 50.6; H, 5.9; N, 9.8; S, 7.5%. ^{13}C-NMR showed absorptions at δ 67 and δ 75.3. From the shielding values summarized by Stothers (11), approximate values of δ 66 and δ 79 were calculated for C^1 and C^2 of this structure. For the alternative positional isomer, δ 50 and δ 81 would be expected.

TLC was carried out on fluorescent plates (Analtech Inc., Newark, Del.) with solvent A, butanol/acetic acid/water (4:1:1, v/v) and solvent B, butanol/ethanol/concentrated NH_4OH/water (4:1:1:1, v/v). Spots were detected by viewing under short wavelength UV, spraying with ninhydrin solution followed by heating at 100°C for a few minutes, and scanning for radioactivity.

Reverse isotope-dilution analyses were carried out by mixing aliquots of extracts of biological fluids with known amounts of unlabeled compounds. After equilibration these were isolated and crystallized to constant specific activity. At least three recrystallizations were carried out for each sample.

Radioactivity was determined in a Triton-toleune medium (10) and counted in a Nuclear-Chicago model Isocap 300 liquid scintillation counter. Counting efficiency was determined by the channels ratio method.

Livers, cannulated at the portal vein, the thoracic inferior vena cava, and the bile duct, were isolated from male Sprague-Dawley rats (250–300 g). The livers were perfused with Krebs-Ringer bicarbonate buffer containing 4.5% bovine serum albumin and 5 mM glucose (150 ml). ^{14}C-Styrene oxide (100 μmol, 1.0 μCi) in acetonitrile (500 μl) was added to the perfusate which was recirculated through the liver for 90 min. Bile was collected either for the total perfusion time or else for fixed time intervals. In the latter case the total bile sample was assayed for radioactivity. Bile collected for the duration of the perfusion experiment was diluted to 1–2 ml with water, counted, and aliquots were analyzed by TLC in solvents A and B. Normally 0.5–0.8 ml bile was collected over 90 min. The perfusate from these experiments was collected and aliquots were assayed for radioactivity after pigments were bleached with 30% H_2O_2 (0.1 ml). Metabolites were extracted from the perfusate as follows: Perfusate (20 ml) was mixed with 5 M $HClO_4$ (1 ml) and centrifuged. The supernatant fluid was mixed with XAD-2 resin (10 g), stirred for 0.5 hr, and filtered. The resin was washed four times with 20-ml portions of water and then four times with 50-ml portions of methanol. At each stage the washings were assayed for radioactivity. The methanolic washings were collected and evaporated under reduced pressure. The residue was dissolved in methanol (10 ml) and analyzed by TLC and reverse isotope-dilution.

Radioactivity bound to protein was determined by the method of Jollow *et al.* (12).

Results

The distribution of radioactivity between the bile and perfusate 1.5 hr after dosing with [8-^{14}C]styrene oxide is shown in table 1. A substantial proportion of the dose in each case was excreted in the bile. A typical time course for the excretion of radioactivity in the bile is shown in fig. 1. In each case maximal excretion was seen after 25 min, with a rapid decline subsequently. Excretion had essentially ceased after 90 min.

Examination of the bile by TLC showed the presence of only one radioactive peak which also coincided with a ninhydrin positive spot (figs. 2 and 3). Synthetic S-(1-phenyl-2-hydroxyethyl) glutathione traveled similarly to the radioactivity in both solvent systems. Reverse isotope-dilution analysis (table 2) established that this compound was the only major radioactive component in the bile.

Preliminary attempts to extract the radioactive components of the perfusate by various organic solvents gave unsatisfactory results. Accordingly, the scheme summarized in fig. 4 was devised. Precipitation of protein from the perfusate with $HClO_4$ led to the loss of 12% of the radioactiv-

TABLE 1

Excretion of radioactivity by isolated perfused liver during 90 min after treatment with [8-^{14}C]styrene oxide (100 μmol/liver)

Experiment	Percentage of Dose in	
	Bile	Perfusate
1	38.6	39.7
2	27.5	69.9
3	40.4	47.7

ity as protein-bound material. The majority (99%) of the radioactivity in the supernatant fraction was adsorbed onto XAD-2 resin but was not completely removed by methanol washing. Other solvents (acetone or methanol-1% acetic acid) failed to remove any further activity from the resin.

TLC analysis of the methanol soluble radioactivity in solvent B showed three radioactive areas on the plate. Spraying with ninhydrin gave no clear indication that the more polar area contained glutathione or related conjugates, because there was a diffuse, poorly separated ninhydrin-positive zone from the origin to R_F 0.3. However, chromatography with authentic reference compounds indicated that styrene glycol, mandelic acid, and S-(1-phenyl-2-hydroxyethyl)glu-

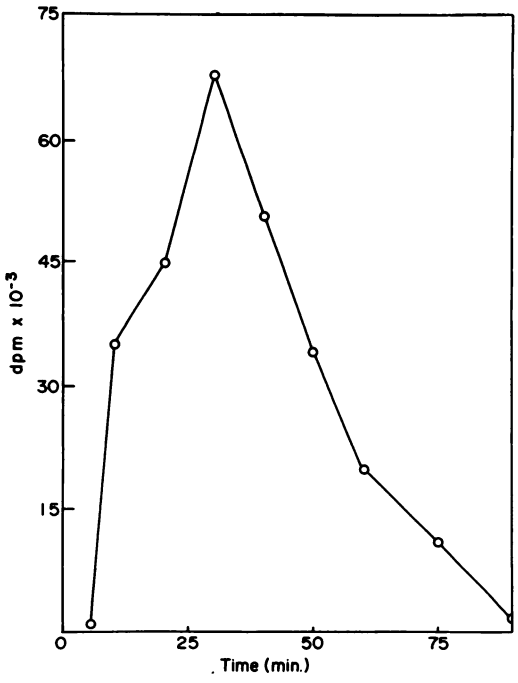

FIG. 1. *Time-course of appearance of radioactivity in bile from ^{14}C-styrene oxide (100 μmol)-treated, isolated, perfused rat liver.*

Data are from a single experiment which gave similar results when repeated.

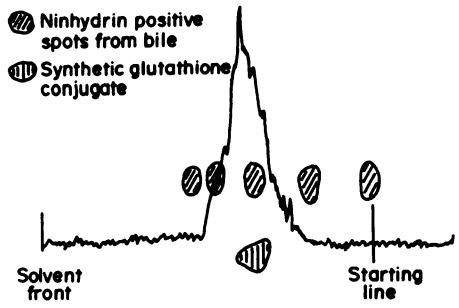

FIG. 2. *TLC (solvent A) of bile from ^{14}C-styrene oxide-treated, isolated, perfused rat liver.*

The trace-line represents the radioactivity distribution on the developed plate.

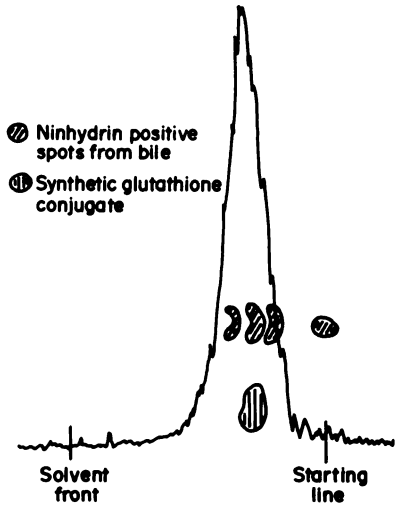

FIG. 3. *TLC (solvent B) of bile from ^{14}C-styrene oxide-treated, isolated, perfused rat liver.*

The trace-line represents the radioactivity distribution on the developed plate.

TABLE 2

Reverse isotope-dilution analysis of metabolites from ^{14}C-styrene oxide-treated, isolated, perfused rat liver

Data are given as percentage of radioactivity of each fraction.

Fraction	Styrene Glycol	Mandelic Acid	S-(1-Phenyl-2-hydroxyethyl) glutathione
		%	
Bile[a]	[b]	[b]	91.6
Perfusate (XAD-2 extract)[c]	35.1, 43.1	31.0, 32.7	21.6, 22.3

[a] Analysis of bile excreted by liver.

[b] None present, as shown by TLC analysis.

[c] Data from perfusate extracts of two livers.

tathione were major components (fig. 5). Reverse isotope-dilution studies verified that these compounds were present as the major components (table 2) of this fraction.

Discussion

The major finding of the present work is that a relatively high proportion of the dosed styrene oxide is converted by the isolated liver to the glutathione conjugate, S-(1-phenyl-2-hydroxyethyl)glutathione. This compound was prepared chemically pure; its ^{13}C-NMR spectrum was consistent with the assigned structure. The metabolism of styrene oxide to the glutathione conjugate was not unexpected, inasmuch as styrene oxide has been used as a convenient substrate for the assay of glutathione S-transferase activity (10, 13, 14). Furthermore, it has been shown that the isolated perfused rabbit lung metabolized styrene oxide to a glutathione conjugate, presumably identical to the conjugate obtained in this work (15). In this earlier work, the presence of a

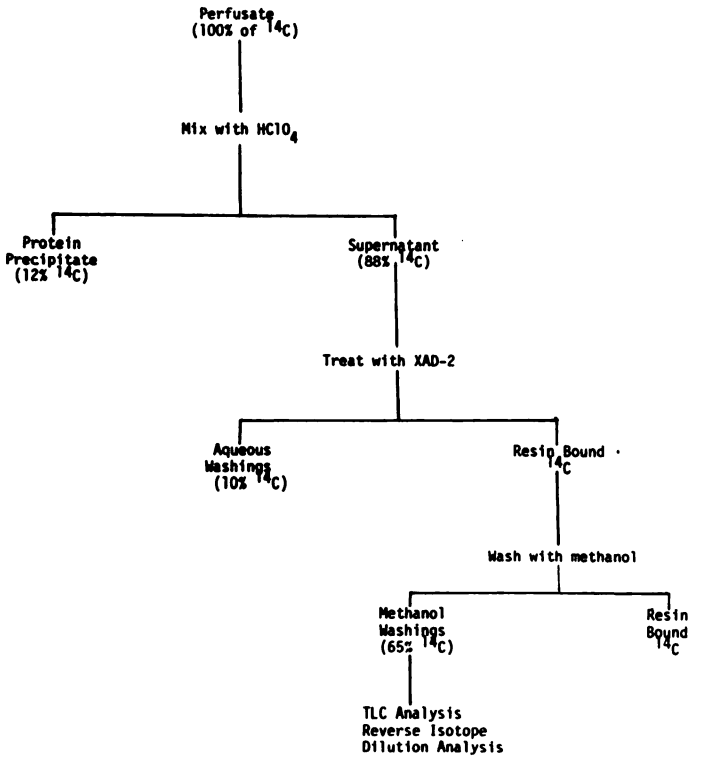

FIG. 4. *Fractionation of perfusate from ^{14}C-styrene oxide-treated, isolated, perfused rat liver.*

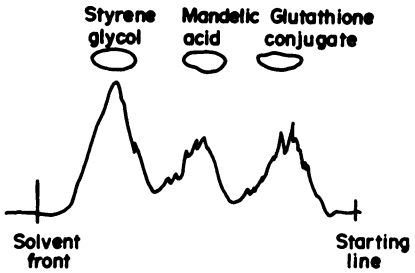

FIG. 5. *TLC (solvent B) of XAD-2 extract from perfusate of ^{14}C-styrene oxide-treated, isolated, perfused rat liver.*

The trace-line represents the radioactivity distribution on the developed plate.

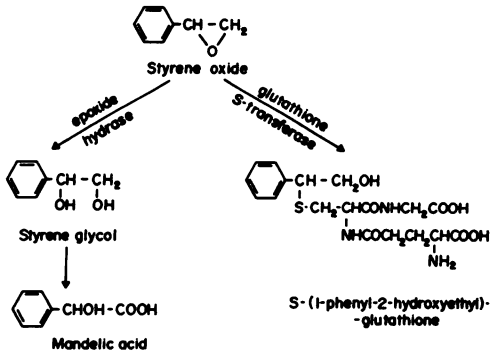

FIG. 6. *Metabolism of styrene oxide in the isolated, perfused rat liver.*

compound which was probably the cysteinylglycine conjugate resulting from enzymatic hydrolysis of the glutathione derivative was also noted. This was not observed as a metabolite in the isolated perfused liver. The biliary metabolite was almost certainly a single compound. Whether any degradation products are formed by hydrolysis of the glutathione conjugate in the liver perfusate is difficult to say. They could only have been minor constituents in the methanol-soluble fraction from the XAD-2 resin. However, it is possible that they could have been in the substantial proportion of radioactivity lost during extraction of the perfusate.

The other metabolites detected in the perfusate were styrene glycol and mandelic acid. Ohtsuji and Ikeda (3) have also detected these compounds in addition to hippuric acid and phenylglycoxylic acid as urinary metabolites in rats given styrene and styrene oxide. In our work styrene glycol and mandelic acid were major metabolites (table 2). The styrene glycol presumably arises from hydrolysis catalyzed by epoxide hydrase and the mandelic acid from oxidation of the glycol by soluble dehydrogenases in liver (fig. 6).

Clearly, at this dose level, styrene oxide is efficiently and approximately equally metabolized by both the hydrase and glutathione-conjugating pathways. This result is not predictable from the *in vitro* data (10), which showed that cytosolic glutathione S-transferase activity, both specific and total, of rat liver was far greater than microsomal epoxide hydrase activity.

At much higher doses than we have used, the Japanese workers (3) only accounted for about 15% of the administered styrene oxide as urinary metabolites. In view of the data presented here, it would be interesting to search for metabolites derived from the glutathione conjugate in the excreta of animals treated with styrene oxide or styrene (*e.g.*, mercapturic acid).

Inasmuch as styrene oxide is a reactive electrophile, we have examined the livers used in these experiments for radioactivity covalently bound to protein. In no case was significant covalent binding detected. This correlates well with the substantial conversion of styrene oxide to soluble metabolites by epoxide hydrase and glutathione transferase. However, only a low level of radioactivity was used in these experiments, so that minor amounts of covalent binding would not have been detected. This point is under further investigation.

Whether similar results (*i.e.*, a similar metabolic profile) would be obtained with styrene itself is an interesting question. It is possible that the relative amounts of metabolites formed from hydration or glutathione conjugation of styrene oxide generated *in situ* by the mixed-function oxidases of the hepatic endoplasmic reticulum may differ from the data presented here, inasmuch as epoxide hydrase is also localized in the endoplasmic reticulum.

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