

Arene oxides in styrene metabolism, a new perspective in styrene toxicity?

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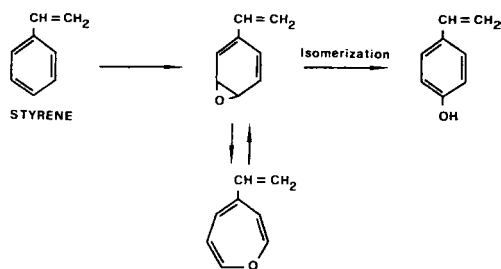
PANTAROTTO, C., FANELLI, R., BIDOLI, F., MORAZZONI, P., SALMONA, M. and SZCZAWINSKA, K. Arene oxides in styrene metabolism, a new perspective in styrene toxicity? *Scand. j. work environ. & health* 4 (1978): suppl. 2, 67-77. The metabolism of styrene was studied in the rat after intraperitoneal administration of the cold and the ¹⁴C-labeled compound. In addition to phenylethylene glycol, mandelic acid, benzoic acid and hippuric acid, phenolic metabolites, namely, 4-vinylphenol, p-hydroxymandelic acid, p-hydroxybenzoic acid, and p-hydroxyhippuric acid, were identified in the urine of the treated animals. These biotransformation products were characterized by mass spectrometry and by comparative thin layer chromatography with standard compounds. Results of covalent binding studies of ¹⁴C-phenylethylene glycol to rat liver microsomal proteins suggest that these phenolic compounds may be formed as a result of chemical rearrangements of unstable arene oxides, reactive intermediates possibly implicated in styrene toxicity.

Key words: arene oxides, covalent binding, mass spectrometry, metabolism, phenylethylene glycol, p-hydroxybenzoic acid, p-hydroxyhippuric acid, p-hydroxymandelic acid, styrene, 4-vinylphenol.

The metabolism of styrene both in man and in laboratory animals is rather well established. The main metabolic pathway is styrene oxidation to phenylethylene glycol (through the formation of the intermediate styrene-7,8-oxide), mandelic acid, phenylglyoxylic acid, benzoic acid and hippuric acid (2, 3, 6, 7, 8, 9, 11, 12, 14). In vivo conversion of both styrene and styrene-7,8-oxide to mercapturic acids and the similar in vitro conjugation of styrene-7,8-oxide with glutathione have been demonstrated (4, 9, 15); a minor metabolism

of styrene in the rat to 1- and 2-phenylethanol and to 4-vinylphenol has also been described (1).

The suggestion concerning the formation of 4-vinylphenol, although based on findings obtained under specific experimental conditions, was of interest because it could involve the intermediacy of an oxide in the 3,4-position, as shown in scheme 1.



Scheme 1. Isomerization of styrene-3,4-oxide to 4-vinylphenol.

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Our interest in arene oxides, both as metabolic intermediates and as mediators of toxic effects, led us to investigate more thoroughly the biotransformation of styrene in rats with particular reference to the formation of phenolic metabolites.

MATERIALS AND METHODS

Standards and reagents

Styrene, styrene-7,8-oxide, phenylethylene glycol, mandelic acid, benzoic acid, and hippuric acid were products of Merck, Darmstadt, Federal Republic of Germany; phenylglyoxylic acid, p-hydroxybenzoic acid and p-hydroxymandelic acid were products of EGA-Chemie, Steinheim/Albuch, Federal Republic of Germany.

^{14}C -styrene (specific activity, 0.5 mCi/mmol, uniformly labeled at the 8-position) was purchased from NEN, Boston, Mass., U.S.A.

^{14}C -phenylethylene glycol was prepared enzymatically, after incubation of the labeled styrene with rat liver microsomes, and further purified by thin layer chromatography. The specific activity for this labeled compound, determined by liquid scintillation counting and by gas chromatography, was also found to be 0.5 mCi/mmol.

Phenobarbital was obtained from Merck, Darmstadt, Federal Republic of Germany, and 3-methylcholanthrene from Sigma, St. Louis, Mo., U.S.A.

The following reagents were used: glucose-6-phosphate, glucose-6-phosphate dehydrogenase, β -glucuronidase-arylsulfatase and nicotinamide adenine dinucleotide phosphate (NADP) (Boehringer, Mannheim, Federal Republic of Germany); N,N-dimethylaniline, methyl iodide, silver oxide, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium dichloride and potassium chloride (Merck, Darmstadt, Federal Republic of Germany).

A 1-M solution of trimethylanilinium hydroxide in methanol was prepared according to the procedure described by

Brochmann-Hanssen and Oke (5), with minor modifications.

All solvents were analytical-reagent grades.

Animals

Male Sprague-Dawley rats (200–220 g) from Charles River, Italy (Calco, Como, Italy), were used.

Hepatic microsomal cytochrome P-450/448 was induced with phenobarbital (80 mg/kg, intraperitoneally, in 0.9% saline once daily for 3 d) or 3-methylcholanthrene (40 mg/kg, intraperitoneally, in corn oil once daily for 3 d).

In vivo metabolism

The animals were housed in individual metabolism cages and were provided with water only. Urine samples were collected 48 h after styrene administration, frozen and kept at -15°C until analysis.

Studies were also carried out in which rats were given the ^{14}C -styrene diluted with cold styrene to a specific activity of 25 $\mu\text{Ci}/\text{mmol}$.

Extraction procedure

Urine samples were incubated with β -glucuronidase-arylsulfatase. The enzyme (0.5 ml) was added to 10 ml of urine adjusted to pH 4.5 with acetate buffer (1 M), and the sample was incubated at 37°C for 12 h. After hydrolysis, 5-ml portions were microextracted with 200 μl of methylene chloride (when volatile metabolites were being dealt with) or extracted twice with 10 ml of ethylacetate. The samples were centrifuged at 3,000 rpm for 20 min at 0°C . We analyzed the methylene chloride extracts by injecting 5 to 10 μl of the organic layer into the gas chromatographic column. The ethylacetate extracts were instead evaporated to dryness at room temperature in a vacuum rotating system. The dry residues were redissolved in 50 μl of methanol, and 1 or 2 μl of this solution were then analyzed gas chromatographically.

Gas-liquid chromatography

Gas-liquid chromatography (GLC) was performed with a Carlo Erba Fractovap mod. G1 gas chromatograph equipped with a flame ionization detector. The column used was a glass tube 2 m long (inner diameter 4 mm), packed with 100–120 mesh Gas Chrom Q coated with 3 % OV 17 (Applied Science Laboratories, State College, Pa., U.S.A.). All newly prepared columns were conditioned at 280°C for 1 h (carrier gas flow rate, 40 ml/min) and then for 4 h at 320°C (without carrier gas flow) and for 24 h at 280°C (carrier gas flow rate, 30 ml/min).

The operating conditions were: injection port heater temperature, 250°C; detector temperature, 250°C. The column oven temperatures are reported in the legends of the figures. During analysis, nitrogen was used as the carrier gas at a flow rate of 30 ml/min. Air and hydrogen flow rates were adjusted to give maximum response.

Mass spectrometry

An LKB mod. 9000 mass spectrometer, coupled with a gas chromatograph, was used under the following conditions: energy of the ionization beam, 70 eV; ion source temperature, 290°C; accelerating voltage, 3.5 kV; trap current, 60 μ A. The samples were introduced by a GLC procedure under the previously described conditions (helium was used as the carrier gas at a flow rate of 30 ml/min; detector, total ion monitor) or by a direct inlet system (DIS) into the ion source of the instrument.

Mass chromatography was performed on an LKB model 2091 B mass spectrometer equipped with computer system model 2130 for data acquisition and calculation.

Thin layer chromatography

Thin layer chromatography was performed on 5 $\times$ 20 cm glass plates precoated with silica gel F-254 (Merck) and developed at room temperature in a solvent system consisting of the organic phase from benzene:ethylacetate:acetic acid:methanol:wa-

ter (14:6:1:3:16). The plates were read under ultraviolet light at 245 and 361 nm and checked for radioactivity on a Packard radiochromatogram scanner.

Preparation of microsomes

Control and phenobarbital- and 3-methylcholanthrene-induced rats were fasted for 24 h before sacrifice. Groups of five similarly pretreated rats were used for the preparation of each batch of microsomes. The animals were killed by decapitation 24 h after the last treatment, and the livers were excised, pooled, and washed with 0.05 M phosphate buffer, pH 7.4. The livers were then minced and homogenized at 0°C in 4 volumes of cold 0.05 M phosphate buffer, pH 7.4, with a Potter-Elvehjem homogenizer with a teflon pestle.

Microsomes were prepared according to Kato and Takayanaghi (10). The protein concentration of the microsomal suspension was determined by the method of Lowry et al. (13), with albumin fraction V as the standard.

Covalent binding of ^{14} C-phenylethylene glycol to microsomal proteins

The incubation mixture in a final volume of 5 ml contained an NADPH-generating system (2.5 μ mol of NADP⁺, 50 μ mol of glucose-6-phosphate and two units of glucose-6-phosphate dehydrogenase), 0.05 M phosphate buffer, pH 7.4, magnesium chloride 5 mM and potassium chloride 0.15 M and about 10 mg of microsomal protein.

After 5 min of preincubation at 37°C the reaction was started by the addition of 10 μ l of an ethanol solution of ^{14} C-phenylethylene glycol to obtain a final substrate concentration of 0.5 mM. Incubation was at 37°C for 120 min in subdued light; the reaction was stopped by the addition of 5 ml ethanol. The samples were centrifuged, the supernatant was discarded, and the precipitate was resuspended in 5 ml of 99 % ethanol. After centrifugation, the precipitate was extracted three times with the following solvent sequence: ethanol 70 %; ethanol 99 %; acetone-chloroform 4:1, v/v; ether and acetone. All the extraction supernatants were tested for radio-

activity. After the last extraction no further radioactivity could be removed from the proteins, even if other solvents were used. This exhaustive extraction thus removed the original ^{14}C -phenylethylene glycol, as well as all metabolites reversibly bound to the proteins. Protein loss was very low during the procedure.

The precipitate was dissolved in 0.5 ml of Soluene[®]-350, Packard, Downers Grove, Ill., U.S.A., and transferred into a counting vial containing 10 ml of a PPO-POPOP solution in toluene. Radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation counter. The values were corrected for quenching by the external standardization method.

RESULTS AND DISCUSSION

Fig. 1 (upper part) shows the gas chromatograms for the methylene chloride ex-

tracts from the urine of controls and styrene-treated rats. A tailing (peak A) was observed that was not present in the chromatogram of the urine extract of control animals. Mass spectrometric analysis of this peak gave the spectrum in fig. 2 (upper spectrum). The molecular ion and base peak at m/e 120 (an increase of 16 mass units with respect to styrene) loses acetylene and gives rise to the peak at m/e 94. Peaks at m/e 91 and 65, which can be assigned the structures of vinylcyclopentadienyl and cyclopentadienyl cations, respectively, arise from the molecular ion and the ion at m/e 94 by loss of a formyl radical group, while ions at m/e 92 and 66 have been explained as shown in scheme 2. The loss of carbon monoxide is a typical fragmentation for phenols and has been explained as proceeding through intermediates of the cyclohexadienone type.

For better structure elucidation, the same urine extracts were treated with tri-

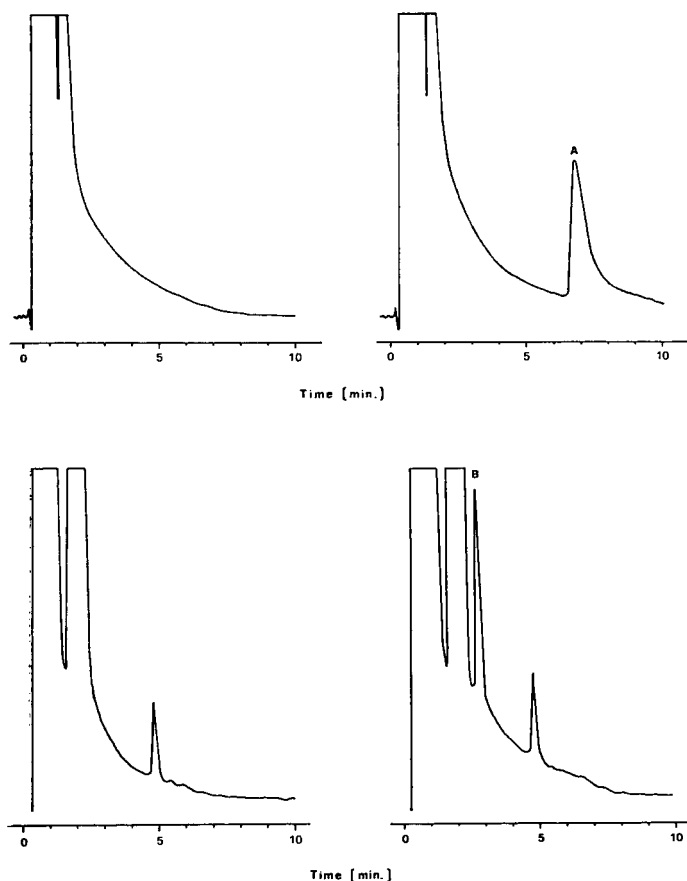


Fig. 1. Upper part: gas chromatograms of the methylene chloride extracts from the urine of controls (on the left) and styrene-treated rats (on the right). Lower part: gas chromatograms of the methylene chloride extracts from the urine of controls (on the left) and styrene-treated rats (on the right) following flash methylation. The analyses were performed with a column temperature of 80°C .

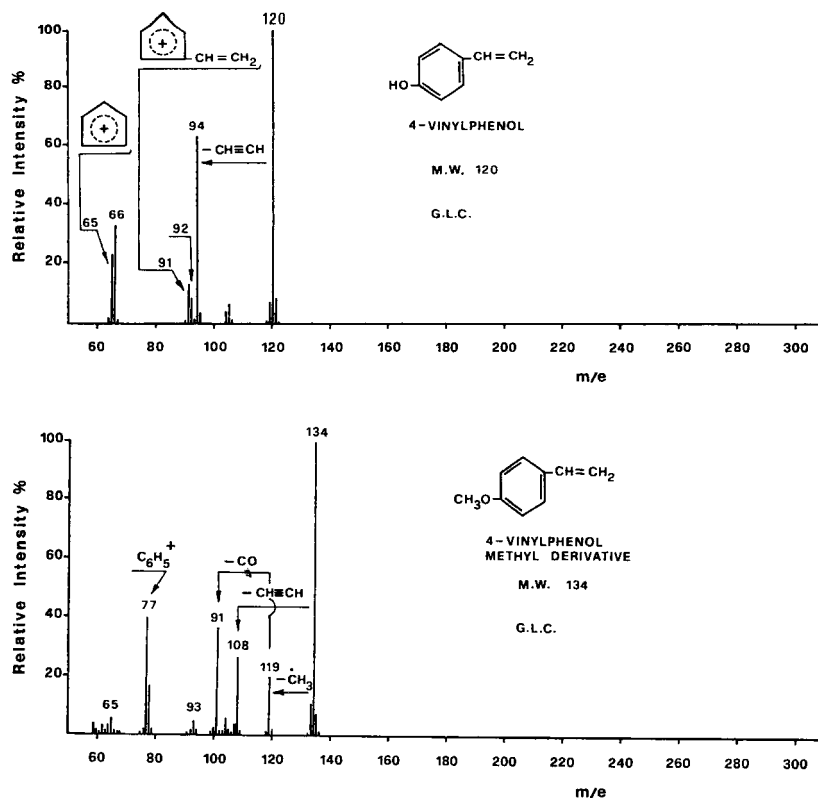
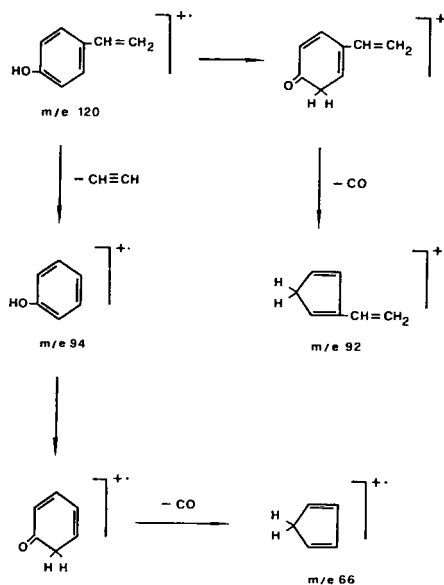


Fig. 2. Mass spectra of 4-vinylphenol and its methylated derivative.

methylanilinium hydroxide, a quaternary ammonium salt used for flash methylations. The resulting chromatograms are shown in fig. 1 (lower part). After on-column methylation, peak B was obtained which, when analyzed by mass spectrometry, gave a spectrum corresponding to the structure of the vinylphenol methyl derivative as shown in fig. 2 (lower spectrum). The molecular ion and base peak at m/e 134 and the other fragments at m/e 119, 108, 91 and 77 are all characteristic of the proposed structure. With this as a basis we therefore assigned this metabolite the structure of vinylphenol, probably in the 4-position.

Table 1 reports the R_f values of some known styrene metabolites, together with those of three other biotransformation products, metabolites I, II and III, isolated from the ethylacetate urine extracts of animals given both cold and ^{14}C -styrene. Fig. 3 shows a gas chromatogram obtained from coinjection with trimethylanilinium hydroxide, the ethylacetate extract from

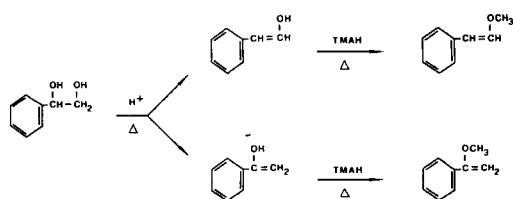


Scheme 2. Rationale for the loss of acetylene and carbon monoxide from 4-vinylphenol.

Table 1. Rf values in the thin layer chromatography of styrene reference compounds and metabolites isolated from rat urine.^a

Compound	Rf value
Phenylethylene glycol	0.45
Mandelic acid	0.06
Phenylglyoxylic acid	0.12
Benzoic acid	0.59
Hippuric acid	0.22
Metabolite I	0.18
Metabolite II	0.52
Metabolite III	0.04

^a Solvent system: organic phase from benzene: ethylacetate:acetic acid:methanol:water (14: 6:1:3:16).



Scheme 3. Gas chromatographic degradation of phenylethylene glycol to 7- and 8-hydroxy-styrene.

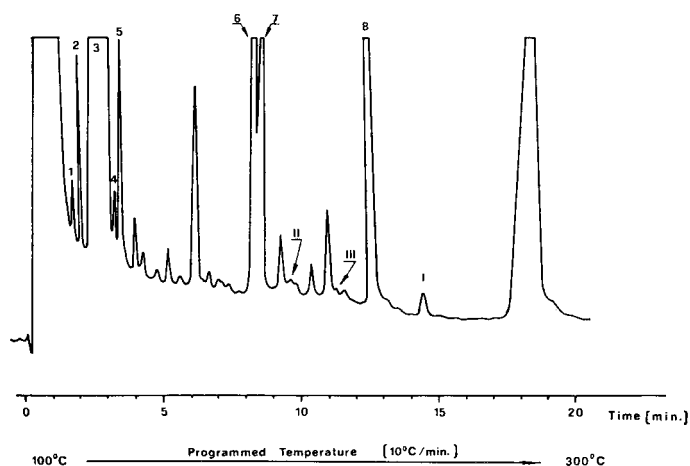


Fig. 3. Gas chromatogram of the ethylacetate extract from the urine of styrene-treated rats. The analysis was performed at a programmed temperature of 100–300°C, the ethylacetate extract being coinjected with trimethylanilinium hydroxide.

the urine of rats treated with cold styrene. An analysis was made at a programmed temperature of 100–300°C. In the chromatogram, the peaks with Arabic numbers correspond to known styrene metabolites, the only exception being peak 3, which is due to N,N-dimethylaniline from trimethylanilinium hydroxide. Therefore, peaks 4, 5, 6, 7 and 8 are the methyl derivatives of 4-vinylphenol, benzoic acid, mandelic acid, phenylglyoxylic acid and hippuric acid. Peaks 1 and 2, identified as 1- and 2-methoxy-1-phenylethylene, were degradation products of phenylethylene glycol, as shown in scheme 3. This degradation is a loss of water, probably catalyzed by the slight acidity of the stationary phase of the gas chromatographic column at a high temperature. The two alcohols formed, in the presence of trimethylanilinium hydroxide, are then readily

converted to the corresponding methyl ethers.

Peaks with Roman numerals in fig. 3 are unidentified metabolites. Fig. 4 shows the mass spectra obtained for metabolite I either through direct introduction of the eluate of the spot at Rf 0.18 into the ion source of the mass spectrometer (upper spectrum) or by GLC after on-column permethylation (lower spectrum). The simple and clear fragmentation and the presence of the cyclopentadienyl cation at m/e 65 were consistent with the structure of a hydroxy derivative of hippuric acid. Metabolite I was therefore identified as hydroxyhippuric acid, probably at the para position.

The presence of this hydroxylated metabolite suggested that hydroxymandelic acid and hydroxybenzoic acid might also be found in the urine. The identification

of both these metabolites was simplified by the availability of pure p-hydroxymandelic acid and p-hydroxybenzoic acid for use as standards. Fig. 5 shows the

mass spectra of p-hydroxyhippuric acid obtained by DIS (upper spectrum) or by GLC after permethylation (lower spectrum). The fragmentation of these com-

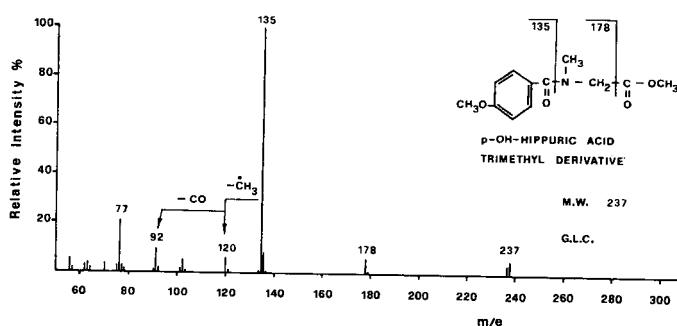
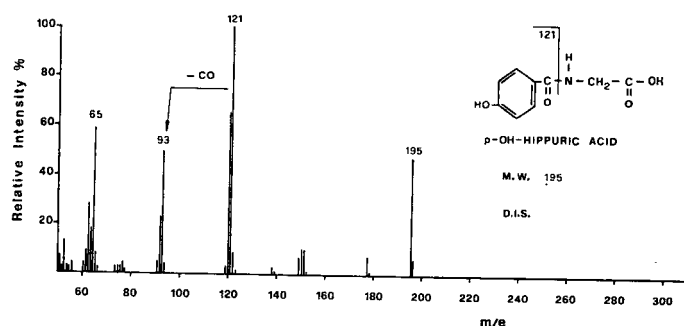


Fig. 4. Mass spectra of p-hydroxyhippuric acid obtained by the direct inlet system and by gas-liquid chromatography after permethylation.

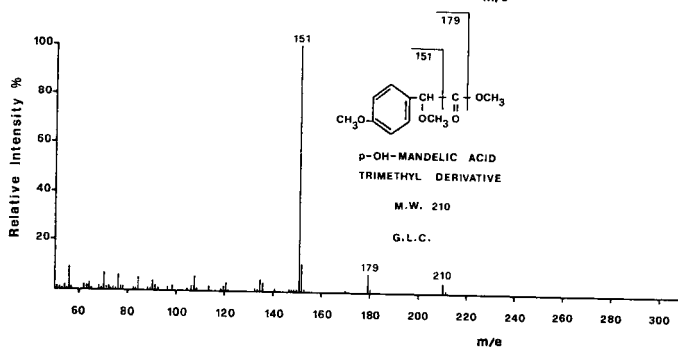
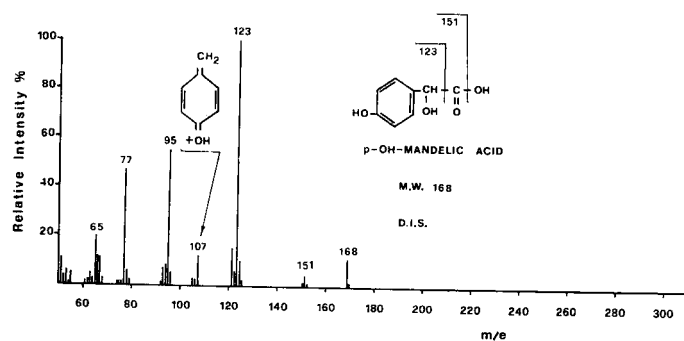


Fig. 5. Mass spectra of p-hydroxymandelic acid obtained by the direct inlet system and by gas-liquid chromatography after permethylation.

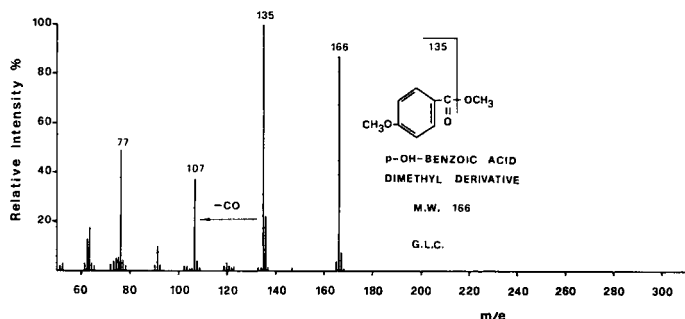
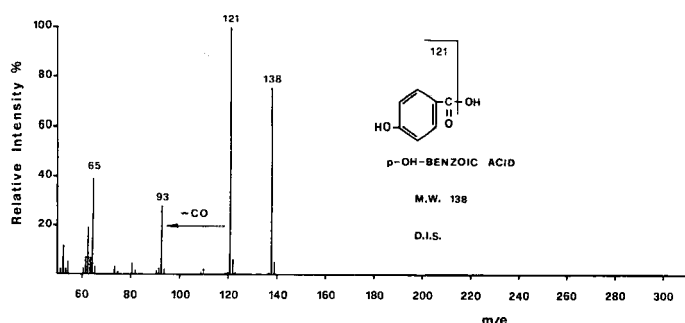


Fig. 6. Mass spectra of p-hydroxybenzoic acid obtained by the direct inlet system and by gas-liquid chromatography after permethylation.

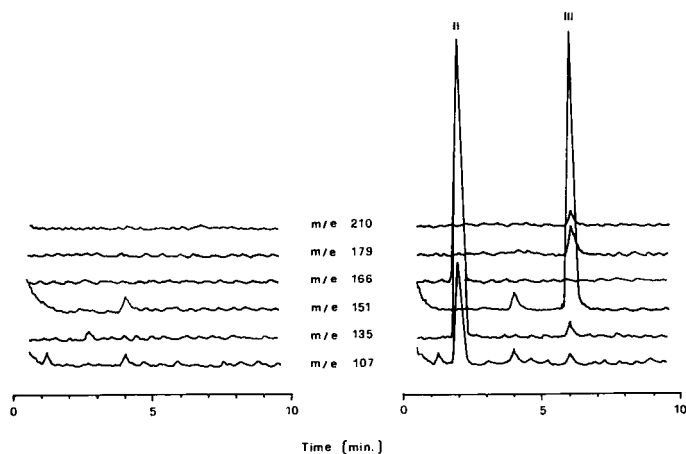
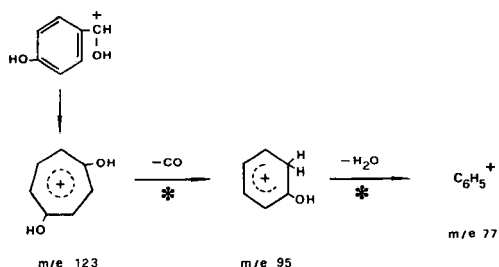


Fig. 7. Mass chromatograms of the ethylacetate extracts from the urine of controls (on the left) and styrene-treated rats (on the right). The analyses were performed with a column temperature of 170°C, the ethylacetate extracts being co-injected with trimethylanilinium hydroxide.



Scheme 4. Rationale for the loss of carbon monoxide and water from the ion at m/e 123.

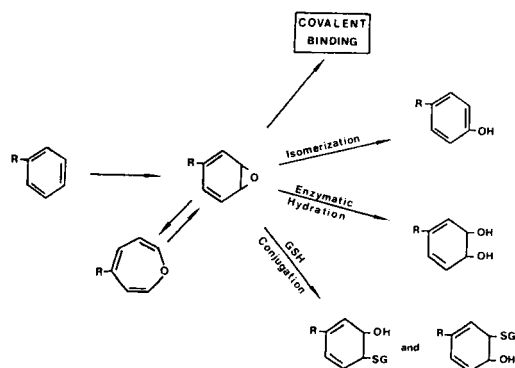
pounds is very simple, as represented in the figure, the ion at m/e 107 being a stable oxonium ion, while the ions at m/e 95 and 77 may arise as shown in scheme 4. The ion at m/e 123, in equilibrium with a more stable seven-membered form, loses carbon monoxide and gives rise to the ion at m/e 95, an hydroxy analog of the benzenium ion, which undergoes dehydration to give the ion at m/e 77. Metastable peaks have been observed that confirm these transitions. Fig. 6 shows the mass spectra of p-hydroxybenzoic acid obtained by DIS

(upper spectrum) or by GLC analysis after derivatization with trimethylanilinium hydroxide (lower spectrum). These spectra are again very simple and characteristic of their respective structures.

On the basis of these findings p-hydroxy-mandelic acid and p-hydroxybenzoic acid were identified in the urine as their methylated derivatives by means of mass chromatography. The mass spectrometer was focused on the ions at m/e 166, 135 and 107 characteristic of the dimethyl derivative of p-hydroxybenzoic acid and on the ions at m/e 210, 179 and 151 characteristic of the spectrum of p-hydroxy-mandelic acid as its trimethyl derivative. Fig. 7 shows the resulting mass chromatogram, which clearly indicates the presence of these two phenolic metabolites in the urine.

Scheme 5 is a proposal for the formation of phenolic metabolites as a result of isomerizations of unstable arene oxides, reactive intermediates which can either bind covalently to cellular macromolecules, conjugate with glutathione, or be hydrated to dihydrodiols.

An indirect demonstration of the intermediacy of arene oxides in the formation of styrene phenolic derivatives is given by our studies on the covalent binding of ^{14}C -phenylethylene glycol to microsomal proteins. The labeled phenylethylene glycol enabled us to distinguish between covalent bindings resulting from epoxida-



Scheme 5. General metabolism of arene oxides.

tion at the 7,8-double bond position from binding determined by oxidation of the aromatic ring at the 3,4-position. Table 2 gives the results of the covalent binding studies. The process, NADPH-dependent, is significantly induced by pretreatment with 3-methylcholanthrene, while no changes in binding were observed in microsomal preparations obtained from the liver of animals pretreated with phenobarbital. Furthermore, binding was greatly inhibited when glutathione was added at a concentration of 1 mM to the incubation mixture.

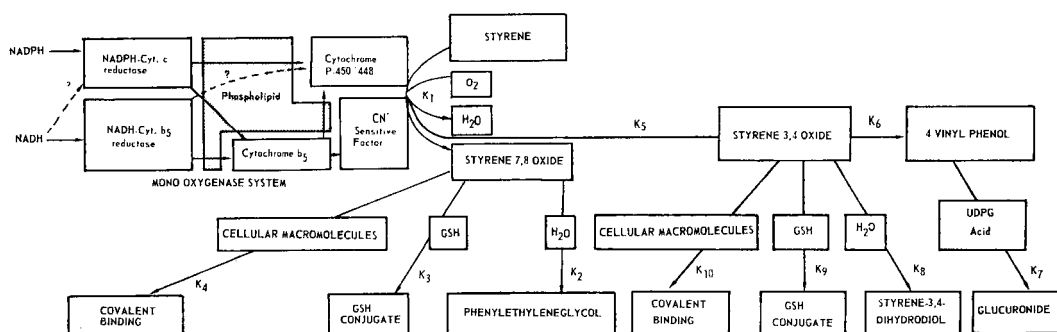
These data indicate that aromatic hydroxylation of styrene is catalyzed by a cytochrome P-448 dependent monooxygen-

Table 2. Irreversible protein binding of ^{14}C -phenylethylene glycol (0.5 mM) catalyzed by rat liver microsomes in the presence of an NADPH-generating system after 120 min of incubation.

Sample	Phenylethylene glycol metabolites irreversibly bound to 1 mg of microsomal protein (d.p.m.) ^a	% radioactivity covalently bound
Boiled control microsomes + NADPH	29 ± 4	0.014
Control microsomes — NADPH	26 ± 1	0.012
Control microsomes + NADPH	73 ± 4	0.034 *
Phenobarbital-induced microsomes + NADPH	85 ± 5	0.040
3-methylcholanthrene-induced microsomes + NADPH	118 ± 9	0.056 **
Control microsomes + NADPH + glutathione (1 mM)	41 ± 3	0.019 ***

^a Each value is the mean ± SE of four determinations.

* $p < 0.01$ compared with boiled control microsomes + NADPH and control microsomes — NADPH; ** $p < 0.01$ compared with control microsomes + NADPH and phenobarbital-induced microsomes + NADPH; *** $p < 0.01$ compared with control microsomes + NADPH.



Scheme 6. Biochemical mechanisms of styrene metabolism and toxicity.

ase. These findings, together with the studies of Watabe and collaborators (16) on the comparative mutagenicity toward *Salmonella typhimurium* TA 100 of styrene, styrene-7,8-oxide and styrene-3,4-oxide, suggest that this new metabolic pathway leading to the formation of phenolic products during styrene biotransformation, even if occurring only to a limited extent, may play an important role as far as the toxicity of this compound is concerned.

Therefore, in conclusion, scheme 6 could be the model for studies aimed at clarifying the biochemical mechanisms involved in styrene toxicity.

ACKNOWLEDGMENTS

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