STUDIES ON METABOLISM AND TOXICITY OF STYRENE. V. THE METABOLISM OF STYRENE, RACEMIC, (*R*)-(+)-, AND (*S*)-(–)-PHENYLOXIRANES IN THE RAT

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Metabolism of styrene, racemic phenyloxirane, (*R*)-(+)-, and (*S*)-(–)-phenyloxiranes in rats has been described. The animals excreted phenylethanediol, mandelic acid, phenylglyoxylic acid, and two regioisomeric mercapturic acids in their urine after the intraperitoneal injection of the phenyloxiranes as well as styrene. The mercapturic acids were identified as *N*-acetyl-*S*-(1-phenyl-2-hydroxyethyl)cysteine (MA-1) and *N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)cysteine (MA-2). The ratios of the mercapturic acids to the other metabolites excreted in the urine were 1 to 1.8 and 1 to 1.5 for styrene and racemic phenyloxirane, respectively. A remarkable stereoselectivity was observed in the excretion of both types of the metabolites when optically active phenyloxiranes were administered. The rate of excretion of the mercapturic acids was 2.5 times higher than that of the other metabolites when the (*R*)-oxirane was injected, but the reverse was the case in the (*S*)-oxirane. The mercapturic acid, MA-1, was excreted at higher rate than the isomer, MA-2, on the administration of styrene and the phenyloxiranes. The most significant regioselectivity in the excretion of MA's was observed when styrene and (*S*)-phenyloxirane were administered.

Keywords—(*R*)-(+)-phenyloxirane; (*S*)-(–)-phenyloxirane; styrene; phenylethanediol; mandelic acid; phenylglyoxylic acid; mercapturic acids; *N*-acetyl-*S*-(1-phenyl-2-hydroxyethyl)cysteine; *N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)cysteine

Phenyloxirane, the major mutagenic metabolite¹⁻³ of the plastic monomer, styrene, in liver microsomes, is detoxicated by two competing enzymes in liver: one is cytosolic glutathione *S*-transferase which conjugates the racemic epoxide with glutathione into two regioisomers, *S*-(1-phenyl-2-hydroxyethyl)glutathione and *S*-(2-phenyl-2-hydroxyethyl)glutathione,^{4,5} and the other microsomal epoxide hydrolase which hydrolyzes the epoxide to phenylethanediol.^{1-3,6,7} The former enzyme catalyzes the detoxication reaction of racemic phenyloxirane at extremely higher rate than does the latter.^{8,9}

Previous studies on the metabolism of styrene and racemic phenyloxirane in rats by several groups of workers showed that the toxic compounds were converted to phenylethanediol,¹⁰ mandelic acid,¹⁰⁻¹³ phenylglyoxylic acid,^{12,13}

and hippuric acid¹⁰⁻¹³ (hydrolysis route), and a study on the metabolism of styrene by another group¹⁴ demonstrated the formation of two mercapturic acids, *N*-acetyl-*S*-(1-phenyl-2-hydroxyethyl)cysteine (MA-1) and *N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)cystein (MA-2), in the ratio 1.9:1 (conjugation route). However, no evidence has been available yet for which metabolic route is predominant in the animal body.

(*R*)-(+)- and (*S*)-(–)-phenyloxiranes, which are both formed from styrene in less stereoselective manner by rat liver microsomes fortified with NADPH, are regiospecifically hydrolyzed by microsomal epoxide hydrolase into (*R*)-(–)- and (*S*)-(+)-phenylethanediols, respectively.¹⁵ (*S*)-Phenyloxirane is hydrolyzed by epoxide hydrolase 4-fold faster than the (*R*)-isomer.¹⁵ These *in vitro* results encouraged us to undertake a study of the

stereochemical course in the *in vivo* metabolism of styrene and optically active phenyloxiranes through the hydrolysis and conjugation routes.

The present investigation provides evidence that styrene, racemic phenyloxirane, and its (*S*)-isomer are metabolized mainly *via* the hydrolysis route, and that the (*R*)-isomer is converted mainly to mercapturic acids *via* the conjugation route. Evidence will also be provided that the administration of either of styrene, racemic phenyloxirane, or its optically pure isomers to rats leads to the predominant formation of the mercapturic acid isomer, MA-1, over the other regioisomer, MA-2.

(*R*)-(-)- and (*S*)-(+)-Phenylethanediols and (*R*)-(+)- and (*S*)-(-)-phenyloxiranes all of which had enantiomeric purities higher than 99% were synthesized by the previously reported methods¹⁵⁾ from (*R*)-(-)- and (*S*)-(+)-mandelic acids (Tokyo Chemical Industry Co., Ltd., Tokyo) with enantiomeric purities higher than 99%. *N*-Acetyl-*S*-(1-phenyl-2-hydroxyethyl) cysteine and *N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)cysteine were synthesized from racemic phenyloxirane according to the method of Seutter-Berlage *et al.*¹⁴⁾

Five male Wistar rats weighing approximately 300 g were injected intraperitoneally with racemic phenyloxirane at a dose of 2 mmol/kg body weight using olive oil as a vehicle (2 ml/kg body weight), and their urine during 24 h were collected for subsequent analysis of the metabolites. The same rats were injected with single doses of the other xenobiotic compounds in order of the (*R*)-epoxide, (*S*)-epoxide, and styrene at weekly intervals in the same manner as described with the racemic epoxide.

For the extraction and determination of the mercapturic acids in rat urine, an aliquot (0.5 ml) of the pooled urine was diluted with an equal volume of 5% aqueous sodium carbonate, and extracted twice with an equal volume of ether to remove neutral and basic materials with lipophilicity. After the urine was acidified with a slight excess of concentrated hydrochloric acid and saturated with sodium chloride, the mercap-

turic acids in urine were extracted with ether. The ethereal extract was concentrated to dryness *in vacuo*, dissolved in 0.1 ml methanol, which contained 2 mg stearic acid as an internal standard, and treated with an ethereal solution of diazomethane for subsequent gas-liquid chromatography-mass spectrometric (GLC-MS) analysis. They were separated on a 5% SE-30 column (3 mm $\times$ 1 m; 200°C, 20 ml/min He) and showed mass spectra superimposable on those of the corresponding authentic specimens: retention time (t_R) at 10.7 min, m/e 297 (M^+), 279 ($M^+ - H_2O$), 236 ($M^+ - H_2O - COCH_3$), 161, 143, 132 (base peak), 107, and 104 for *N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)cysteine methyl ester; and t_R at 12.8 min, m/e 279 ($M^+ - H_2O$), 267, 236 ($M^+ - H_2O - COCH_3$), 161, 143, 104 (base peak, $PhCHCH_2$), and 91 for *N*-acetyl-*S*-(1-phenyl-2-hydroxyethyl)cysteine methyl ester.

For the hydrolysis of the conjugates of phenylethanediol, mandelic and phenylglyoxylic acids, the urine acidified with 1/10 vol. concentrated hydrochloric acid was heated at 85°C for 1 h. After cooling to room temperature and being saturated with sodium chloride, the hydrolysate was extracted with twice volumes of ethyl acetate. In order to collect the organic acids, the ethyl acetate layer was shaken with 1/4 vol. 10% aqueous sodium carbonate. After the acidification of the alkaline solution by the addition of a slight excess of concentrated hydrochloric acid, the free acids were re-extracted with twice volumes of ethyl acetate following saturation of the aqueous phase with sodium chloride. To the ethyl acetate layer, concentrated to 0.5 ml at atmospheric pressure, was added 0.1 ml methanol containing 1 mg *p*-chlorobenzoic acid as an internal standard. The acids obtained were then methylated with an ethereal solution of diazomethane for subsequent GLC-MS analysis. For the assay of phenylethanediol, ethyl acetate layer obtained from the acid-hydrolyzed urine as described above, was gently concentrated to 0.5 ml at atmospheric pressure. The glycol was derived into the acetonide and determined as previously described.³⁾ The methyl esters and the acetonide,

separated on a 15% diethylene glycol succinate column (3 mm $\times$ 2 m; 180°C and 125°C for mandelic and phenylglyoxylic acids and phenylethanedione, respectively; 20 ml/min He), showed molecular ion peaks and characteristic fragment ion peaks identical with those from the authentic specimens: t_R at 7.5 min, m/e 164 (M^+), 106 (base peak, PhCHO), 105, and 77 for methyl phenylglyoxylate; t_R at 10.8 min, m/e 166 (M^+), 107 (base peak, $M^+ - COOCH_3$), and 77 for

methyl mandelate; t_R at 7.6 min, m/e 178 (M^+), 163 ($M^+ - CH_3$), 148 ($M^+ - CH_2O$), 120, and 72 (base peak) for the acetonide of phenylethanedione.

The per cent ratios of phenylethanedione, mandelic acid, phenylglyoxylic acid, MA-1, and MA-2 in urine of rats to styrene administered were 0.9, 8.1, 34.0, 16.2, and 7.2, respectively. When racemic phenylloxirane was administered, the per cent ratios were almost the same as those; 1.5, 7.5, 38.0, 18.5, and 13.5, respectively. Phenylglyoxylic

FIG. 1. Metabolic Hydrolysis and Mercapturic Acid Conjugation of (R)-(+)- and (S)-(-)-Phenylloxiranes in Rats

Values under the metabolites represent molar percent ratios to (R)-(+)- or (S)-(-)-phenylloxiranes administered.

acid was the most abundant of the three non-sulfured metabolites, and the next mandelic acid. The total ratios of non-sulfured metabolites excreted were very high; 43.0 and 47.0 to administered styrene and racemic phenyloxirane, respectively.

Styrene and racemic phenyloxirane were converted into the non-sulfured metabolites rather than the mercapturic acids: the molar ratios of the formers to the latters were 1.8 for styrene and 1.5 for racemic epoxide. The results are not coincident with those from the *in vitro* studies, which showed racemic phenyloxirane was detoxicated by cytosolic glutathione *S*-transferase 20⁸⁾ or 80⁹⁾ times faster than by microsomal epoxide hydrolase. With respect to these two detoxication reactions, Ryan and Bend¹⁶⁾ demonstrated that the two enzymes catalyzed the reactions to the same extent using isolated perfused rat livers. They also showed that the more the applied dose of the epoxide increased, the more hydrolysis products, phenylethanediol and mandelic acid, were excreted. These facts suggest that the unexpected small amounts of MA-1 and MA-2 excreted in urine of rats injected with styrene or racemic phenyloxirane would be due to the rapid decrease in hepatic glutathione contents by the conjugate formation with the epoxide formed or administered. A content of glutathione in liver, 3–6 $\mu\text{mol/g}$ liver,^{17,18)} seems to be insufficient for glutathione conjugation of all of the epoxide administered at a dose of 2 mmol/kg body weight. Since epoxidation of the styrene vinyl side chain by hepatic microsomes fortified with NADPH proceeds at an extremely high rate,^{1–3)} administration of styrene to rats may also lead to the depletion of hepatic glutathione. It has already been reported that glutathione was depleted from the liver of animals receiving well-known inducers of hepatic necrosis, acetaminophen¹⁹⁾ or bromobenzene²⁰⁾ whose reactive metabolites are conjugated with glutathione and finally transformed to readily excreting mercapturic acids without toxicity.

The present investigation provides the first evidence for stereoselective metabolism of

phenyloxirane isomers in whole animals. The percent ratios of mercapturic acids excreted in urine of rats were 55.2 and 21.9 to (*R*)- and (*S*)-phenyloxiranes administered, respectively. Excretion of MA-1 and MA-2 was 2.5-fold more than the total amounts of the non-sulfured metabolites when (*R*)-phenyloxirane was administered, whereas the reverse was the case in the (*S*)-isomer (Fig. 1). These *in vivo* results are coincident with the previous results obtained by *in vitro* experiments that the (*S*)-epoxide is hydrolyzed by hepatic microsomal epoxide hydrolase 4 times faster than the (*R*)-isomer.¹⁵⁾ The total urinary excretion of the metabolites determined was 65.0, 79.0, 77.6, and 75.6% for styrene, racemic, (*R*)-, and (*S*)-phenyloxiranes, respectively. This indicates styrene to be metabolized in large part *via* phenyloxirane in the rat. The epoxide would play a large part in the metabolism of the hydrocarbon because urinary benzoic acid and hippuric acid which are known to be derived from metabolically formed phenylglyoxylic acid in the animal body^{10–13)} were not included in the data.

As demonstrated in this study, a very large portion of styrene injected was metabolized *via* mutagenic phenyloxirane into non-toxic compounds. However, 2-vinylphenol,²¹⁾ 4-vinylphenol,^{13,21–23)} 4-hydroxymandelic acid²³⁾, and 4-hydroxyphenylglyoxylic acid²³⁾ have also been detected as minor metabolites from the urine of rats injected with styrene. This strongly suggests the biological formation of 1-vinylbenzene 1,2-oxide and 3,4-oxide in the animal body. Although these highly reactive epoxides are assumed to be minor metabolites, the study on these epoxides are of importance because of their extremely higher mutagenic activities to *Salmonella typhimurium* TA 100 than that of phenyloxirane.²¹⁾

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