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Stereochemistry in the oxidative metabolism of styrene by hepatic microsomes

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The incubation of styrene, one of the principal precursors in the industrial production of polymer materials, with liver microsomes in the presence of NADPH leads to the formation of phenyloxirane as an intermediate, which is rapidly metabolized further into phenylethanediol [1–6]. The first step of this metabolic pathway, epoxide formation, is catalyzed by liver monooxygenases, and the formed chemically reactive epoxide, phenyloxirane, has been noticed as a toxic metabolite since it was reported to be a mutagen in the *Salmonella typhimurium* TA strains used in the Ames testing system [5, 7–11], and suggested to be a skin tumorigen in mice [12]. It has a reactivity to bind covalently to rat liver macromolecules, especially to protein, *in vivo* and *in vitro* [5, 13]. The second step is hydrolysis of the epoxide formed, which is catalyzed by microsomal epoxide hydrolase. In this step the toxic epoxide can be hydrolyzed to the chemically and toxicologically less active glycol, phenylethanediol. Phenyloxirane is one of the most frequently used substrates for the assay of microsomal epoxide hydrolase activity [14–16]. Except the previous demonstrations that the enzymatic phenylethanediol formation from racemic phenyloxirane was initiated by the introduction of a hydroxy group specifically to the 3-position of the oxirane ring [17] and also that rabbit liver microsomal epoxide hydrolase had almost no preference for either *R*- and *S*-phenyloxirane, producing phenylethanediol of much less than 1% optical purity from racemic phenyloxirane [17]. It is of interest that rats given styrene excreted optically active mandelic acid in urine [18]. This is suggestive of the stereo-selective formation of phenylethanediol from styrene in the animal body since the acid proved to be yielded directly from the diol [18].

As to cyclic system, a number of stereochemical studies have been carried out on hepatic microsomal epoxidation and epoxide hydrolysis, e.g. epoxidation of benzo[*a*]pyrene [19, 20], naphthalene [17], cholesterol [21], pregnenolone [22], and 1,3,5(10),16-estratetraene [23], and hydrolysis of benzo[*a*]pyrene 4,5-, 7,8-, and 9,10-epoxides [20], benzo[*a*]pyrene 7,8-diol-9,10-epoxides [19, 20], naphthalene oxide [17], cholesterol 5,6-epoxides [21], pregnenolone 5,6-epoxides [22], 2,3-epoxysteroids [24], estratetraenol epoxides [23], cyclohexene oxide [17], and 4-*tert*-butyl-1,2-epoxycyclohexanes [25]. However, nothing is known of stereochemistry, including absolute configurations of oxygenated carbons, in the microsomal epoxidation of acyclic olefins especially regarding the chirality formation from an achiral molecule and in their hydrolysis especially regarding the chiral selectivity in enantiomeric epoxides although stereoselectivity in hepatic microsomal hydrolysis has been partially shown with a few epoxides such as stilbene oxides [26], mono-substituted stilbene oxides [27], and 9,10-epoxystearates [28]. It is of importance to investigate the optical selectivity in the microsomal oxidative metabolism of the vinyl side chain of sytrene as a model substrate in these respects. In connection with this, previous workers provided tentative evidence that there was little difference between rates of hepatic microsomal hydrolysis of the *R*- and *S*-enantiomers in racemic phenyloxirane [17].

A promising approach to this problem could be to separate and determine *R*-, *S*-, and racemic phenyloxiranes since it appears from the results of the previous investigations that the epoxides yielded from the hydrocarbon by hepatic microsomes in the presence of NADPH are completely converted during incubations at intervals longer than 30 min into phenylethanediol [5, 6] without inversion of the absolute configurations at 2-position of the oxiranes [17]. For approaching to the problem, we synthesized *R*- and *S*-phenyloxiranes and *R*- and *S*-phenylethanediols with purities all higher than 98 per cent and established an HPLC (high performance liquid chromatography) method for the resolution of the enantiomers of phenylethanediol as their diastereoisomeric di-*R*-(+)-MTPA (α-methoxy-α-trifluoromethylphenylacetic acid) esters. In the present communication, we wish to report that there exists a remarkable difference in the rates of microsomal hydrolysis of phenyloxirane enantiomers and also that hepatic microsomal epoxidation of the sytrene vinyl group proceeds with low stereoselectivity to yield *S*-phenyloxirane at slightly higher rate than the *R*-isomer. Finally, we will also provide evidence for the equivalent mutagenicity of *R*- and *S*-phenyloxiranes toward *Salmonella typhimurium* TA strains.

R- and *S*-phenyloxiranes used in the present investigation were synthesized as follows: *R*-(−)- and *S*-(+)-phenylethanediols derived from *R*-(−)- and *S*-(+)-mandelic acids (Tokyo Chemical Industry Co. Ltd., Tokyo) with enantiomeric purities more than 98 per cent, respectively, by the reduction with lithium aluminium hydride in the standard manner [29], were reacted with a 0.9 molar ratio each of *p*-toluene-sulfonyl (tosyl) chloride in dry pyridine at 0° for 15 hr and at room temperature for 1 hr. Resulting *R*- and *S*-phenyltosyloxymethylcarbinols (m.p. 70° each) were

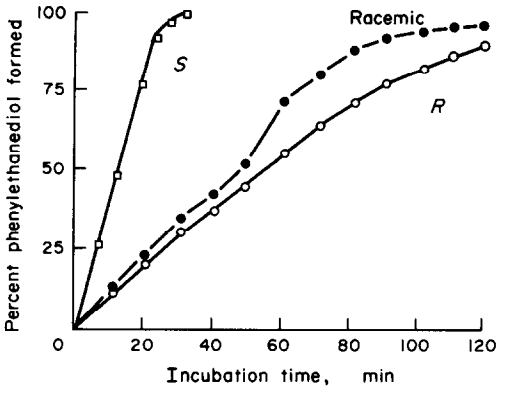

Fig. 1. A time course study of hepatic microsomal hydrolysis of *R*-, *S*-, and racemic phenyloxiranes. Each oxirane (2 mM) dissolved in acetone (2 per cent v/v) was incubated at 37° in an air tight flask with washed microsomes (0.95 mg protein/ml) isolated from male Wistar rat liver and suspended in 0.1 M phosphate buffer, pH 7.4. The reaction was terminated by the addition of an aqueous NaOH solution. Phenylethanediols formed were extracted with ethyl acetate after the removal of the substrates from the mixtures by the extraction with n-hexane followed by the saturation of the aqueous phases with sodium chloride. *R*-(from *R*-phenyloxirane) and *S*-(from *S*-phenyloxirane)-phenylethanediols were separated and determined at 254 nm by HPLC. Phenylethanediol from racemic phenyloxirane was represented as summed amounts of its *R*- and *S*-enantiomers formed.

separated from di-tosylates of phenylethanediols by silica gel column chromatography in mixtures of benzene and increasing ratios of acetone (2 to 10 per cent) and then from 2-phenyl-2-tosyloxyethan-1-ols by recrystallizations from n-hexane-ether. *R*-(+)-Phenyloxirane (b.p. 75°/7 mmHg, $[\alpha]_D^{20}$ +6.7° (*c* 4.9)) and *S*-(−)-phenyloxirane (b.p. 75°/7 mmHg, $[\alpha]_D^{20}$ −6.8° (*c* 4.9)) were obtained by the treatment of the recrystallized *R*- and *S*-monotosylates, with an alcoholic potassium hydroxide solution, respectively, followed by distillation.

The previously reported incubation conditions and method for the extraction of metabolites [5, 6] were applied to the present investigation of hepatic microsomal hydrolysis of *R*-, *S*-, and racemic phenyloxiranes. For the assay of phenyloxirane formed during the incubation with rat liver microsomes, the alkalinized incubation mixture was extracted with n-hexane for subsequent gaschromatographic analysis. For the assay of phenylethanediol formed, the residual aqueous phase was washed with n-hexane and extracted with ethyl acetate after being saturated with sodium chloride. After the solvent was carefully evaporated on an oil bath at 100°. The residue obtained was dissolved in 0.2 ml of an acetonide reagent, composed of 5 ml dry acetone and a drop of 60 per cent perchloric acid. The solution was left stand for 30 min and then agitated after the addition of 50 mg of finely powdered anhydrous sodium carbonate to decompose the acid catalyst. The acetonide formed and phenyloxirane were determined by a JEOL Model JGC-29KFP gaschromatograph equipped with a 15 per cent DEGS column coated on Shimalite, 60-80 mesh, 2 m × 3 mm, at a column temperature of 134°. The enzymatically formed phenylethanediol enantiomers were separated and determined by HPLC with monitoring at 254 nm after derivatization as their *R*-(+)-MTPA (Aldrich Chemical Co. Ltd., Milwaukee, Wis.) diesters by the method of Dale and Mosher [30]. The di-*R*-(+)-MTPA esters of *R*- and *S*-phenylethanediols had retention times of 24 and 28 min, respectively, on a silica gel column (μPorasil) in n-hexane containing 2.5 per cent tetrahydrofuran as a developing solvent mixture (2.0 ml/min). The diastereoisomeric diesters in the effluent from the HPLC column were separately collected and identified with authentic specimens by mass spectroscopy. Male adult Wistar rat liver microsomes (0.95 mg protein/ml) suspended in 0.1 M phosphate buffer, pH 7.4, hydrolyzed *R*- and *S*-phenyloxiranes (2 mM each) at relative rates of 1 (12.5 nmoles/mg protein/min): 4 specifically to *R*- and *S*-phenylethanediols, respectively, indicating that hydrolysis of their oxirane rings by microsomal epoxide hydrolase proceeded by the highly selective (at least 98 per cent) introduction of a hydroxy group from water to their 3-(unsubstituted)carbons as had been demonstrated by the previous workers [17] (reported more than 90 per cent) with racemic phenyloxirane as the substrate in ^{18}O-enriched water. A kinetic study of the enzymatic reaction indicated that a marked difference existed between the epoxy enantiomers in affinity for epoxide hydrolase : $K_m 29 \times 10^{-6}$ M and V_{max} 11.6 nmoles/mg protein/min for the *R*-oxirane and $K_m 155 \times 10^{-6}$ M and V_{max} 44.1 nmoles/mg protein/min for the *S*-oxirane obtained from the double reciprocal plot in the range 125–500 × 10^{-6} M substrate concentrations vs rates of phenylethanediol formation.

Racemic phenyloxirane yielded both *R*- and *S*-phenylethanediols when incubated under the same conditions as mentioned above. A comparative study of time courses in the enzymatic hydrolysis of *R*-, *S*-, and racemic phenyloxiranes showed that the racemate was hydrolyzed with a bi-phase reaction profile, i.e. at an earlier stage of the

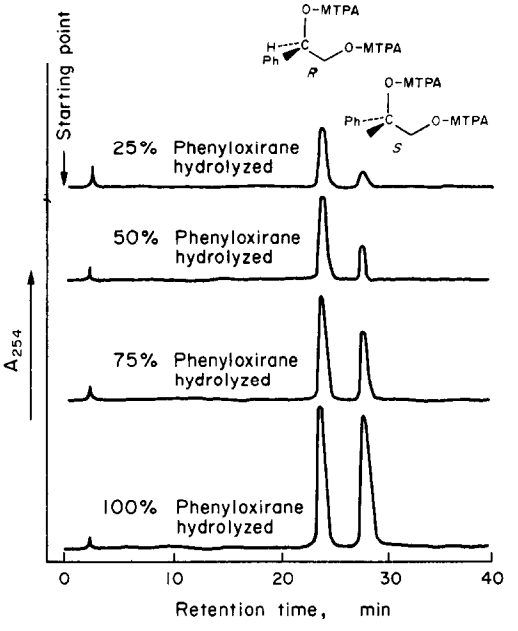

Fig. 2. HPLC separation of diastereoisomeric MTPA diesters of *R*- and *S*-phenylethanediols formed during incubations of racemic phenyloxirane with rat liver microsomes. Racemic phenyloxirane (2 mM) was incubated under the same conditions as described in Fig. 1 for 15 min with various amounts of microsomes: 2.0, 3.7, 4.4, and 6.0 mg microsomal protein/ml for reaching 25, 50, 75, and 100 per cent hydrolysis of the substrate, respectively. A Laboratory Data Control Milton Roy liquid chromatograph Model Constametric II G equipped with a μPorasil column (3.9 × 30 cm) and a uv monitor was used. n-Hexane containing 2.5 per cent tetrahydrofuran was used as a developing solvent mixture (2 ml/min).

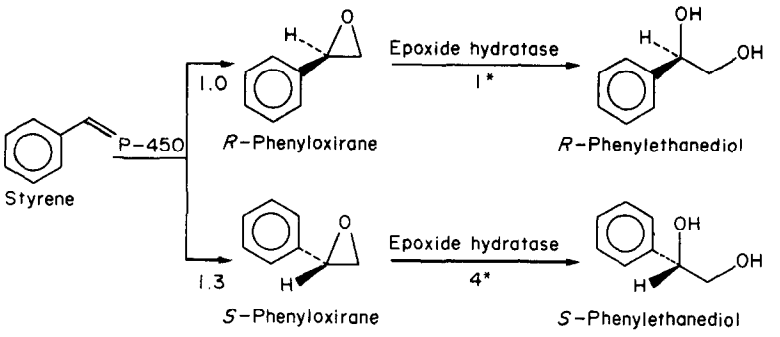

Fig. 3. Stereoselective metabolism of sytrene into phenylethanediols via phenyloxiranes by rat liver microsomes. Numerals under arrows represent relative reaction rates: 2.67 nmoles/mg protein/min for 1.0 and 12.5 nmoles/mg protein/min for 1*.

reaction, it yielded phenylethanediols at a slightly higher rate than the *R*-oxirane, and then the reaction rate increased rapidly after about one half of the racemate was consumed (Fig. 1). *R*-Phenylethanediol was formed at much higher rates than the *S*-diol especially at earlier stages of the reaction of the racemic oxirane, and at the final stage the ratio of both diols was equal (Fig. 2). The higher rate of the *R*-diol formation from the racemate was reasonably interpreted by an inhibitory effect of its precursor, *R*-phenyloxirane, on the enzymatic hydrolysis of the *S*-epoxy enantiomer because the *R*-oxirane in the racemate could be assumed from its smaller K_m value to have a higher affinity for the microsomal hydrolase than the other enantiomeric counterpart.

For the investigation of stereochemistry in the microsomal epoxidation of the styrene vinyl group, the hydrocarbon was incubated at pH 7.4 for 60 min with rat liver microsomes in the presence of an NADPH-generating system under the previously reported conditions [5, 6]. Under these incubation conditions, phenyloxirane formed from styrene has been demonstrated to be completely hydrolyzed to phenylethanediol by the action of epoxide hydrolase [5, 6] whose activity was almost unchanged during prolonged incubations by becoming higher relatively to the decreasing activity of monooxygenase according to the progress in microsomal lipid peroxidation [31]. After the absence of phenyloxirane in the incubation mixture was checked at the end of the reaction by GLC as previously reported [6], phenylethanediols were determined as di-*R*-(+)-MTPA esters in the same manner as described above. Both *R*- and *S*-phenylethanediols were detected from the mixture. Data indicated that *S*-phenyloxirane was formed 1.3 times faster than the *R*-isomer in the monooxygenation of styrene. That no further oxidative metabolism occurred with the biologically formed phenylethanediol enantiomers during incubations was confirmed by the quantitative recovery of separately added enantiomers and racemate from the complete incubation mixtures without styrene. Thus, stereochemistry in the metabolism of styrene to phenylethanediol via phenyloxirane by rat liver microsomes has been established as summarized in Fig. 3.

Liver microsomes from male young adult Wistar rats pretreated with 3-methylcholanthrene or phenobarbital enhanced the rates of epoxidation of sytrene and hydrolysis of phenyloxirane [2]. However, no appreciable alteration was observed in the *R* and *S* ratio of phenylethanediols formed from styrene as well as in the relative rate of the *R*- to *S*-diols formed from enantiomeric phenyloxiranes.

Racemic phenyloxirane has been demonstrated to be an intrinsic mutagen toward bacteria [5, 7–11] as well as a tumorigen toward mouse skin on painting [12]. In order to know whether there is any difference in bacterial mutagenesis between this kind of small-sized enantiomers or not, a comparative study was made by using *Salmonella typhimurium* TA strains according to the method of McCann *et al.* [32]. In the absence of a PCB-induced rat liver S9 (9000 *g* supernatant fraction) and an NADPH-generating system, they were all mutagenic to the strain TA 100 but not to any of the other strains, TA 98, TA 1537, and TA 1538. Dose-response curves indicated that there was no substantial difference between the *R*- and *S*-oxiranes as well as between the racemate and the enantiomers. *S. typhimurium* TA 100 showed 766 of induced His^+ revertant colonies/plate at a dose of 4 μmoles/plate of each phenyloxirane.

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Combined effects of guanidinoethanesulfonate, a depletor of tissue taurine levels, and isoproterenol or methoxamine on rat tissues

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Taurine, a ubiquitous amino acid constituent of mammalian tissues, has been implicated as having a physiologic role in cardiac function. A definitive mechanism for taurine in cardiac tissue, however, has thus far eluded investigators. It has been proposed that taurine is an antiarrhythmic agent capable of reversing drug-induced electrical abnormalities [1, 2]. Various studies have also suggested that taurine might alter cardiac function by modulating calcium utilization [3–5]. Guidotti *et al.* [3] have demonstrated that taurine increases the positive inotropic effects of strophanthin in the perfused heart. Likewise, Dietrich and Diacono [4] and Iwata and Fujimoto [5] have also reported that taurine potentiates the inotropic effect of ouabain. Moreover, it has been demonstrated that taurine increases calcium binding rate and calcium content of the sarcoplasmic reticulum [6]. Evidence of a function for taurine in cardiac tissue has been reported by Chovan *et al.* [7] who suggested that low-affinity taurine binding sites appear to regulate calcium levels in the cardiac sarcolemma.

Huxtable *et al.* [8] have postulated that part of the difficulty in defining a mechanism for taurine is the inability of the investigator to modify the *in vivo* taurine content of cardiac tissue. In addressing this problem, they [8] demonstrated that the rat can be partially depleted of its tissue stores of taurine by the addition of 1.0% guanidinoethanesulfonate (GES) to the drinking water. GES, a structural analogue of taurine, depletes tissue taurine content by inhibiting taurine transport. However, while the extent of taurine depletion was considerable, for example after 4 weeks of treatment cardiac tissue contained only 20 per cent of control taurine levels, liver, 24 per cent and cerebellum, 33 per cent, additional treatment with GES did not deplete taurine content below these values [8]. In addition, results from our laboratory [9] have demonstrated that isoproterenol (ISO) and methoxamine (MOX), both sympathomimetic agents, diminish the taurine content of cardiac tissue by 30–40 per cent and enhance the concentrations of taurine in blood by 70–100 per cent.

In the present communication, the combined effects of GES and ISO or GES and MOX on rat tissues were investigated.

Male Sprague–Dawley rats weighing 250–290 g were used in all experiments. The animals had free access to water and food and were fed Ralston Purina Rodent Laboratory Chow No. 5001. Experimental animals were maintained on drinking water containing 1.5% GES for 35 days. GES was synthesized and characterized as described by Huxtable *et al.* [8].