Biochemical Pharmacology, Vol. 32, No. 5, pp. 777–785, 1983.
Printed in Great Britain.

0006-2952/83/050777-09 \$03.00/0

STUDIES ON METABOLISM AND TOXICITY OF STYRENE—VI. REGIOSELECTIVITY IN GLUTATHIONE *S*-CONJUGATION AND HYDROLYSIS OF RACEMIC, *R*- AND *S*-PHENYLOXIRANES IN RAT LIVER

TADASHI WATABE, NAOKI OZAWA and AKIRA HIRATSUKA
Laboratory of Drug Metabolism and Toxicology, Department of Hygienic Chemistry, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji-shi, Tokyo 192-03, Japan

(*Received* 15 *June* 1982; *accepted* 26 *August* 1982)

Abstract—Rat liver cytosol converted phenyloxirane enantiomers regioselectively to glutathione *S*-conjugates. *R*-(+)-Phenyloxirane was converted to *S*-(1-phenyl-2-hydroxyethyl)glutathione (conjugate **1**) and *S*-(2-phenyl-2-hydroxyethyl)glutathione (conjugate **2**) (ratio 6.1:1), and *S*-(−)-phenyloxirane to conjugates **1** and **2** (ratio 1:32). Racemic phenyloxirane was converted to conjugates **1** and **2** (ratio 1.8:1). The conjugates were separated by HPLC on an octadecylsilicone column and identified with synthetic specimens whose structures were assigned by ^{13}C NMR spectrometry.

R-(+)-, *S*-(−)- and racemic phenyloxiranes were hydrolyzed to *R*-(−)-, *S*-(+)- and racemic phenylethanediols by microsomal epoxide hydrolase without inversion of absolute configurations of their benzylic carbons. *R*-(+)-Phenyloxirane had much smaller K_m and V_{max} than the *S*-(−)-oxirane did. The *R*-(+)-oxirane potentially inhibited the microsomal hydrolysis of the *S*-(−)-oxirane and was preferentially hydrolyzed when the racemic oxirane was used as the substrate. Microsomal monooxygenase oxidized styrene to *R*-(+)- and *S*-(−)-phenyloxiranes (ratio 1.3:1), and the ratio was little changed by the pretreatment of the animal with phenobarbital, 3-methylcholanthrene and polychlorinated biphenyls.

Phenyloxirane, the major mutagenic metabolite in the oxidative metabolism of the plastic monomer, styrene (ethenyl benzene), by hepatic microsomes [1–4], has been most frequently used as a standard substrate for the assay of microsomal epoxide hydrolase [5–7] and soluble glutathione *S*-transferase activities in various tissues [8–10]. The enzymes play key roles in the inactivation of reactive epoxides formed as ultimate forms by microsomal monooxygenases from a wide variety of exogeneous arenes and olefins having carcinogenicity, mutagenicity or toxicity [11, 12]. In the liver of various species of animals, phenyloxirane is preferentially inactivated by both enzymes [10, 13–15]. Much attention has recently been paid to the metabolism of this standard substrate *in vivo* as well as *in vitro* in order to know the nature of the enzymatic reactions in view of stereochemistry involving regioisomerism and enantiomerism [14, 16, 17].

Only a little information, however, has been available as yet for the mode of introduction of the glutathione sulphydryl group not only to the oxirane carbons of phenyloxirane, but also to those of other xenobiotic epoxides in the metabolic conjugation reaction. With respect to the oxirane fused to the ring system, the enzymatic conjugation reaction has been shown to occur regiospecifically with dihydronaphthalene oxide [18, 19], naphthalene oxide [20, 21] and cholesterol α-epoxide [22] and regioselectively with benzo[*a*]pyrene, 4,5-oxide [23].

Phenyloxirane was first reported to react with glutathione specifically at the benzylic carbon under both enzymatic and non-enzymatic conditions to yield a single conjugate [14]. Later, tentative evidence was provided for the formation of regioisomers of the conjugate [17]. The latter work, however, failed to separate them but showed that the chromatographically single and identical conjugates isolated from enzymatic and non-enzymatic reaction media gave phenethyl alcohol and methylphenylcarbinol on treatment with Raney nickel. In a previous communication, we reported the biological formation of regioisomeric phenyloxirane–glutathione *S*-conjugates and the inadequacy of the Raney nickel desulphuration method for the determination of the sulphur-bearing atom of an epoxide–glutathione *S*-conjugate [24]. Both of the purely isolated regioisomers yielded phenyloxirane as the major desulphuration product which was then readily hydrogenated to phenethyl alcohol and methylphenylcarbinol.

The recent *in vivo* studies on the metabolism of styrene, phenyloxirane, and its enantiomers have demonstrated that they are excreted as regioisomeric mercapturic acids into urine [25–27].

With respect to the hydrolysis of phenyloxirane, it has been demonstrated by mass spectrometry (MS)* that rabbit liver microsomal hydrolysis of the racemic epoxide proceeds highly regioselectively in ^{18}O-enriched water to yield phenylethanediol with

---

* Abbreviations: ^{13}C NMR, carbon-13 NMR; ^{1}H NMR, proton NMR; HPLC, high-pressure liquid chromatography; MS, mass spectroscopy; MTPA, α-methoxy-α-trifluoromethylphenylacetic acid; NADP, nicotinamide-adenine dinucleotide phosphate; K_m, Michaelis constant; V_{max}, maximum velocity; S_{N2}, substitution, nucleophilic, bimolecular.

at least more than 90% of the ^{18}O at the non-benzylic carbon when 18% of the substrate was consumed [16]. However, the very recent work from our laboratory [28] has shown that, in rat liver microsomes, the *R*-isomer of racemic phenyloxirane is preferentially hydrolyzed to the *S*-isomer by epoxide hydrolase until about 50% of the substrate is consumed. Therefore, the regioselectivity of microsomal hydrolysis of *S*-phenyloxirane is still obscure.

The present paper provides evidence for the extremely high regioselectivity in the glutathione *S*-conjugation of phenyloxirane enantiomers by cytosolic glutathione *S*-transferase and their regiospecific hydrolysis to the corresponding enantiomeric phenylethanediols with a remarkable difference in rate by microsomal epoxide hydrolase. Evidence will also be provided for a small difference in the microsomal formation of the epoxide enantiomers from styrene.

MATERIALS AND METHODS

Materials. Racemic phenyloxirane and styrene, which were redistilled under reduced pressure before use, racemic phenylethanediol, 3-methylcholanthrene, sodium phenobarbiturate and polychlorinated biphenyls (Kanechlor 400) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan) and NADP, glucose 6-phosphate and glucose-6-phosphate dehydrogenase from Oriental Yeast Co. Ltd (Tokyo, Japan). Glutathione, over 99% pure and free from its oxidized form, was obtained from Yamanouchi Pharmaceutical Co. Ltd (Tokyo, Japan). *R*-(+)-MTPA (99% pure) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Other reagents used were all reagent grade.

R-(+)- and *S*-(−)-phenyloxiranes with enantiomeric purity higher than 99% each were synthesized as follows: *R*-(−)- and *S*-(+)-phenylethanediols derived from *R*-(−)- and *S*-(+)-mandelic acids (Tokyo Chemical Industry Co. Ltd, Tokyo, Japan) with enantiomeric purities higher than 99%, respectively, by the reduction with lithium aluminium hydride by an earlier method [29], were reacted with *p*-toluenesulphonyl (tosyl) chloride in dry pyridine at 0° for 15 hr and then at room temp for 1 hr. In order to retard the previously reported formation of 2-phenyl-2-tosyloxyethanol as a side product leading to the decrease in enantiomeric purities of resulting phenyloxiranes [29], tosyl chloride was used at a 0.9 molar ratio to each phenylethanediol (1.45 M in pyridine). The reaction mixtures were diluted with ether and washed successively with 1 N HCl and water for the removal of pyridine and the unreacted diols. The residues obtained on the evaporation of the solvent from the ethereal solutions which were dried over anhydrous sodium sulphate were subjected to silica gel column chromatography carried out in benzene and acetone.

From the chromatographic fractions obtained by the elution of the column with benzene and acetone (9:1) were isolated *R*- and *S*-phenyltosyloxymethylcarbinols as thin-layer chromatographically pure crystals. They were recrystallized from *n*-hexane and ether to give prisms (68 and 65% yields for the *R*- and *S*-tosylates, respectively), m.p. 70° each; MS *m/z*: 262 [$M^+ - CH_2O$], 120, 107 (base peak), 92, 91, 79 and 77; NMR: δ (ppm) [$(CD_3)_2CO$]: 2.42 (s, 3 H), 4.05 (d, $J = 5$ Hz, 2 H), 4.92 (t, $J = 5$ Hz, 1 H), 7.31 (s, 5 H), 7.40 (d, $J = 8$ Hz, 2 H), 7.75 (d, $J = 8$ Hz, 2 H); i.r.: ν_{max} (cm^{-1}) (KBr): 3520 (νOH), 1590, 1490, 1340 ($\nu_{as}SO_2$), 1170 (ν_sSO_2), 1090, 1020, 960, 920, 870, 820, 770, 700; u.v.: λ_{max} (nm) (ε) (EtOH): 262 (650), 224 (6370). *R*-(+)-Phenyloxirane ($[\alpha]_D^{20}$ +6.8° (c 4.9, acetone)) and *S*-(−)-phenyloxirane ($[\alpha]_D^{20}$ −6.7° (c 4.9, acetone)) were obtained from the recrystallized (*R*)- and (*S*)-monotosylates as follows: the tosylates (0.5 M each) were dissolved in 15% (w/v) methanolic KOH solutions and left to stand for 2 hr at room temp. The reaction mixtures were diluted with 5 vols of ether and washed twice with a small vol. of water. From the organic phases, separated and dried over anhydrous sodium sulphate, ether was removed by fractional distillation using a fractional column, and the residual solutions were distilled under reduced pressure to collect the oxiranes. Overall yields of *R*-(+)- and *S*-(−)-phenyloxiranes were 56 and 48%, respectively.

R-(+)-MTPA diesters of *R*-(−)-, *S*-(+)- and racemic phenylethanediols were prepared by the method of Dale and Mosher [30].

Liver subcellular fractions and pretreatments of rats. Livers isolated from male Wistar rats weighing 180–200 g were immediately perfused and homogenized with chilled isotonic KCl solution. The 3-vol. homogenate obtained was centrifuged at 9000 *g* for 20 min. A post-mitochondrial separated fraction was centrifuged at 105,000 *g* for 60 min, and the supernatant fraction carefullly taken. Microsomal pellets were resuspended in 20 vols of isotonic KCl and resedimented under the same conditions as mentioned earlier. The washing procedure was repeated again, and the twice washed microsomes were suspended in 0.1 M phosphate buffer, pH 7.4, so that 1 ml of the suspension was equivalent to 1 g of liver or 9.6 mg of protein. The soluble supernatant fraction separated was dialyzed for 20 hr at 0–2° through a Visking membrane against 1500 vols of 0.1 M phosphate buffer, pH 7.4, and then its vol. was adjusted with the same fresh buffer so that 1 ml was equivalent to 0.25 g liver or 7.8 mg protein. Protein contents of the microsomes and the soluble supernatant fraction were determined by the method of Lowry *et al.* [31]. Pretreatment of the animal with phenobarbital, 3-methylcholanthrene and polychlorinated biphenyls was carried out by the previously reported method [32].

Incubation conditions and isolation of metabolites. For the investigation of glutathione conjugation reactions, glass-stoppered flasks containing the dialyzed soluble supernatant fraction (final concn of protein 0.62 mg/ml) and glutathione (4 mM) in 0.1 M phosphate buffer, pH 7.4, were preincubated for 10 min at 37°, and incubations continued for a further 5 min after the addition of *R*-(+)-, *S*-(−)- or racemic phenyloxirane (2 mM) in acetone [2% (v/v)]. For the termination of the reaction, the reaction vessel was chilled and immediately shaken with 2 vols of ether to remove the unchanged substrate. From the aqueous layer separated by centrifugation, dissolved ether was removed by aspiration at room temp. The residual aqueous solution was poured onto an Amberlite XAD-2 column (four bed vols to the solution). The column was washed with water (three bed vols to the column) and eluted with 50% (w/w) aqueous methanol (three bed vols). The aqueous methanolic effluent was collected into a single fraction, and then the solvent was evaporated under reduced pressure.

For the investigation of epoxide hydrolysis reactions, glass-stoppered flasks containing twice washed microsomes (0.2–1.0 mg protein/ml) in 0.1 M phosphate buffer, pH 7.4, were preincubated for 10 min at 37°, and incubations were continued for a further 5–120 min after the addition of *R*-(+)-, *S*-(−)- or racemic phenyloxirane (0.125–2 mM) in acetone [2% (v/v)]. The reaction was terminated by the addition of 5 N NaOH so that it made a final concn of 1 N. The mixture was extracted with 1/3 vol. of *n*-hexane containing naphthalene as an internal standard for subsequent direct GLC analysis of the unchanged phenyloxiranes. The aqueous layer separated by centrifugation was washed twice with 3 vols of *n*-hexane for the complete removal of the remaining substrate. Extraction and condensation of phenylethanediols from the aqueous layer for subsequent analysis by GLC as their acetonides were carried out as previously reported [4]. Aliquots of the condensates were also used for the derivatization of phenylethanediols to their *R*-(+)-MTPA diesters for subsequent analysis by HPLC.

For the investigation of the vinyl side chain oxidation in styrene, incubations of the substrate with microsomes in the presence of an NADPH-generating system and isolation of phenylethanediols formed were carried out under the same conditions as previously reported [4].

Chromatography. HPLC was carried out on a Milton Roy liquid chromatograph Model Constametric II G equipped with a u.v. monitor (254 nm). GLC was carried out on a Shimadzu Model GC-4CM equipped with a hydrogen-flame ionization detector. A glass column (4 mm × 2 m) packed with 15% diethylene glycol succinate coated on 60–80 mesh Chromosorb W was used at a flow rate of 40 ml nitrogen/min and at column temps of 115° for the determination of phenyloxirane (retention time 10 min) in the presence of naphthalene (11.4 min) as an internal standard and 125° for the acetonide of phenylethanediol (11.2 min) in the presence of naphthalene (8.1 min). The injection port temps were kept 30° higher than those of the columns. TLC was carried out on glass plates (5 × 20 cm^2) coated with silica gel containing an inorganic fluorescent reagent and a binder (Wakogel B5-F, Wako Pure Chemical Industries Ltd) or on plastic sheets coated with cellulose F_{254} (E. Merck, Darmstadt, F.R.G.).

Spectrometry. ^{13}C NMR spectra were recorded at 25 MHz on a JEOL Model TX 100 system magnetic resonance spectrometer. U.v. spectra were recorded on a Hitachi Model 557 spectrophotometer. Mass spectra of *R*-(+)-MTPA diesters and mono-tosyl esters of phenylethanediols were recorded by the direct inlet system on a Hitachi Model RMU-7L mass spectrometer. The ionization energy applied was 70 eV, and the samples were heated at 130°. GLC mass spectra were recorded on a Shimadzu-LKB Model 9000 gas chromatograph mass spectrometer equipped with a column packed with 15% diethylene glycol succinate coated on 60–80 mesh Chromsorb W (3 mm × 3 m). The flow rate of helium as carrier gas was 20 ml/min. The temp for the injection port, the separator and the ion source chamber were kept at 250, 280 and 310°, respectively. The ionization energy applied was 20 eV.

RESULTS

Separation and structural assignment of regioisomers of phenyloxirane–glutathione S*-conjugates*

A synthetic glutathione conjugate preparation obtained as an amorphous solid from racemic phenyloxirane by the method of Ryan and Bend [14] showed a single spot on cellulose powder plates in various solvent mixtures, which was visualized by ninhydrin or by a u.v. lamp (254 nm), e.g. at R_f 0.40 in *n*-butanol–water–acetic acid (4:1:1). The synthetic specimen, however, was demonstrated by HPLC to consist of at least two conjugates appearing at 9 min (conjugate **1**) and 11.5 min (conjugate **2**), their peak ratio being 1:1.4 (Fig. 1). They were separately eluted from the HPLC column, isolated as white solids after the evaporation of the solvent, and then recrystallized twice from aqueous methanol to yield colourless powders. They had the same R_f values as that of the specimen before separation in the cellulose powder thin-layer chromatograms.

Structural assignment of conjugates **1** and **2** was carried out by ^{13}C NMR spectrometry. The assigned structures were Ph–CH(SG)–CH$_2$OH [*S*-(1-phenyl-2-hydroxyethyl)glutathione] for conjugate **1** and Ph–CH(OH)–CH$_2$SG [*S*-(2-phenyl-2-hydroxyethyl)glutathione] for conjugate **2** (Table 1). From the signals of the phenylethane carbons C_1 and C_2

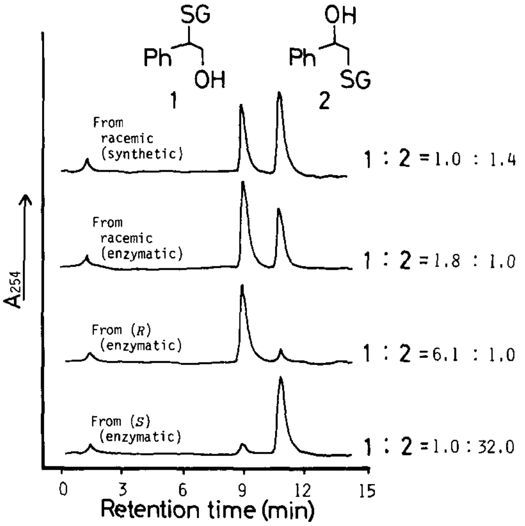
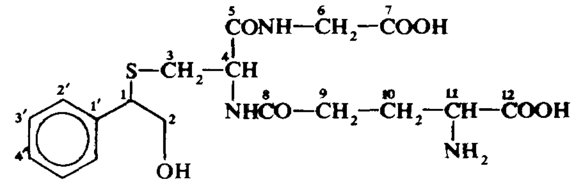
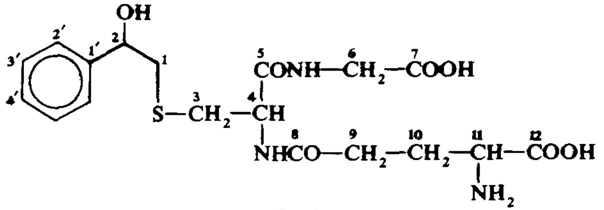


Fig. 1. HPLC separation of glutathione *S*-conjugates formed from racemic, *R*-(+)- and *S*-(−)-phenyloxiranes by the catalytic action of sodium hydroxide or hepatic glutathione *S*-transferase. HPLC was performed at 30° on an octadecylsilicone column (μBondapak C_{18}, 10 μ particle size, 3.9 mm × 30 cm) in 1 ml/min of methanol–water–acetic acid (20:180:1).

Table 1. ^{13}C chemical shifts of the phenyloxirane–glutathione conjugates*


Conjugate 1


Conjugate 2

Compound carbon	Chemical shifts (δ ppm from TMS) Conjugate 1	Conjugate 2
1	53.7 (d)	40.2 (t)
2	65.3 (t)	73.2 (d)
3	33.2 (t)	33.9 (t)
4	53.7 (d)	53.7 (d)
5	172.3 (s)	172.7 (s)
6	42.8 (t)	42.3 (t)
7	174.5 (s)	173.9 (s)
8	175.0 (s)	175.0 (s)
9	31.9 (t)	31.8 (t)
10	26.8 (t)	26.6 (t)
11	52.3 (d)	52.0 (d)
12	173.9 (s)	172.9 (s)
1′	140.1 (s)	142.5 (s)
2′	128.7	126.7
3′	129.6	129.3
4′	129.3	128.7

* Racemic phenyloxirane (0.3 M) was reacted for 3 hr at room temp with glutathione (0.3 M) and NaOH (0.6 M) in ethanol and water (5:8). The conjugates isolated from the reaction mixture on an XAD-2 column after neutralization of the mixture with hydrochloric acid were subjected to HPLC carried out under the same conditions as described in Fig. 1. The separated conjugates were recrystallized from aqueous methanol. Spectra were recorded with 0.1 M solutions in deuterated water. Signals (multiplicity) were assigned by means of partially decoupled off-resonance spectra. t, triplet; d, doublet; s, singlet.

each conjugate was not found to be contaminated with the other regioisomer. The signals due to C_1 (40.2 ppm, triplet on the partial off-resonance) and C_2 (73.2 ppm, doublet) of conjugate **2** were not found in the spectrum of conjugate **1**. On the other hand, the signal due to C_2 (65.3 ppm, triplet) of conjugate **1** was not found in the spectrum of conjugate **2**.

Regioselective glutathione S-conjugation of phenyloxirane enantiomers by rat liver cytosol

Racemic phenyloxirane (2 mM) was briefly (5 min) incubated at pH 7.4 with a dialyzed soluble supernatant fraction (0.62 mg protein/ml) of a rat liver homogenate in the presence of glutathione (4 mM). After the reaction was terminated by the immediate removal of the substrate from the medium into ether, the biologically formed conjugates were adsorbed on an XAD-2 column and eluted with aqueous methanol. The high-pressure liquid chromatogram obtained under the same conditions as used for the separation of the synthetic conjugates indicated that conjugates **1** and **2** were both formed at rates of 62.2 and 34.6 nmoles/mg protein/min (Fig. 1). Under these conditions, 15% of the racemic substrate was consumed in the presence of the hepatic soluble fraction, but the substrate consumption during incubations was less than 0.7% when the subcellular fraction was boiled or omitted. The biologically formed conjugates which were separated by HPLC had the same R_f values as those for the synthetic ones in the cellulose powder thin-layer chromatograms visualized with ninhydrin or by the u.v. lamp.

R-(+)-Phenyloxirane with an enantiomeric purity higher than 99% yielded conjugate **1** preferentially to conjugate **2** (ratio 6.1:1), where 1 for the ratio represented 12 nmoles/mg protein/min when incubated under the afore-mentioned conditions (Fig. 1). On the other hand, *S*-(−)-phenyloxirane with an enantiomeric purity higher than 99% yielded conjugate **2** preferentially to conjugate **1** (ratio 32:1) under the same conditions, where 1 for the ratio represented 1.2 nmoles/mg protein/min (Fig. 1). Data indicated that the ratio (1.5:1) of summed amounts of conjugates **1** to **2** enzymatically formed from *R*-(+)- and *S*-(−)-phenyloxiranes was similar to that from racemic phenyloxirane.

Similar regioselectivities were observed in the non-enzymatic phenyloxirane–glutathione *S*-conjugation reactions when *R*-(+)- and *S*-(−)-phenyloxiranes were used as substrates: ratios of conjugates **1** to **2** formed were 4:1 and 1:19 for the *R*-(+)- and *S*-(−)-oxiranes, respectively, both at pH 7.4 and in a 0.1 N aqueous sodium hydroxide solution. They were carried out at the same concns of the substrates and glutathione as those used in the biological reactions. The alkaline-mediated reactions were so fast that their rates could not be determined at 37°.

Hydrolysis of phenyloxirane enantiomers by rat liver microsomes

Hepatic microsomal hydrolysis of *R*-(+)-, *S*(−)- and racemic phenyloxiranes was examined in order to investigate stereoselectivity in enzymatic epoxide hydrolysis. Phenylethanediols formed were determined either by GLC after the derivatization to acetonides when only reaction rates were required or by HPLC after the derivatization to *R*-(+)-MTPA diesters when enantiomeric separation was required.

S-(−)-Phenyloxirane was hydrolyzed by microsomes to phenylethanediol 4 times as fast as *R*-(+)- or racemic phenyloxiranes. A kinetic study of the enzymatic reaction indicated that there existed a marked difference in affinity to epoxide hydrolase between *S*-(−)- and *R*-(+)-phenyloxiranes: K_m 155×10^{-6} M and V_{max} 44.1 nmoles/mg protein/min for the *S*-(−)-oxirane and K_m 29×10^{-6} M and V_{max} 11.6 nmoles/mg protein/min for the *R*-(+)-oxirane, obtained from the double-reciprocal plot in the range of 125×10^{-6}–500×10^{-6} M substrate concn vs rate of formation of phenylethanediols. This strongly sug-

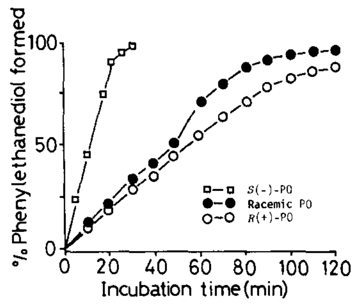


Fig. 2. Time courses of enzymatic hydrolysis of *R*-(+)-, *S*-(−)- and racemic phenyloxiranes (PO) in rat liver microsomes. Phenyloxiranes (2 mM each) were incubated with microsomes (1.0 mg protein/ml) at pH 7.4.

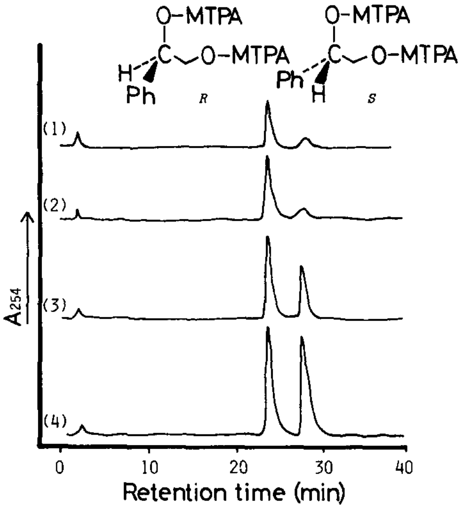


Fig. 3. HPLC separation of diastereoisomeric *R*-(+)-MTPA diesters of *R*-(−)- and *S*-(+)-phenylethanediols formed from racemic phenyloxirane during incubations with rat liver microsomes. Incubations were carried out under the same conditions as described in Fig. 2. Chromatograms represent: (1) 25%, (2) 50%, (3) 75%, and (4) 100% racemic phenyloxirane hydrolyzed respectively. HPLC was carried out at 20° on a silica gel column (μPorasil, 5 μ particle size, 3.9 mm × 30 cm) in 2.5% tetrahydrofuran–*n*-hexane (2 ml/min). They were monitored at 254 nm.

gested that in racemic phenyloxirane the *R*-(+)-oxirane with smaller K_m could inhibit the enzymatic hydrolysis of the *S*-(−)-enantiomer. A comparative study of time courses in the enzymatic hydrolysis of *S*-(−)-, *R*-(+)- and racemic phenyloxiranes showed that the racemate was hydrolyzed with a biphasic reaction profile, i.e. at an earlier stage of the reaction it yielded phenylethanediol 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. 2).

In enzymatic hydrolysis, *R*-(−)-phenylethandiol was formed specifically from *R*-(+)-phenyloxirane, and *S*-(+)-phenylethanediol also specifically from *S*-(−)-phenyloxirane. *R*-(+)-MTPA diesters of the enzymatically formed *R*(−)- and *S*-(+)-phenylethanediols were separated and determined by HPLC with a sufficient difference in retention time (Fig. 3). Mass spectra of these diastereoisomeric diesters were almost superimposable on each other: *m/z* 337 [M^+ − $OOCC(OCH_3)(CF_3)Ph$], 189 (base peak), 119, 104, 77 and 69. Racemic phenyloxirane yielded both *R*-(−)- and *S*-(+)-phenylethanediols by microsomes. At earlier stages of the enzymatic reaction, the racemate yielded the *R*-(−)-diol at a higher rate than the *S*-(+)-diol. The preferential formation of the *R*-(−)-diol to the *S*-(+)-diol from the racemic oxirane continued until most of the *R*-(+)-oxirane in the substrate was consumed (about 50% hydrolysis), and then the rate of *S*-(+)-diol formation increased. That agrees with the reaction profile as illustrated in Fig. 2.

Epoxidation of styrene by rat liver microsomes

For the investigation of stereochemistry in the microsomal epoxidation of the styrene vinyl group, the hydrocarbon was incubated for 60 min with rat liver microsomes in the presence of an NADPH-generating system. Under these incubation conditions, phenyloxirane formed from styrene by monooxygenase has been demonstrated to be completely hydrolyzed to phenylethanediol by the action of

Table 2. Conversion of styrene to phenylethanediol enantiomers via phenyloxiranes by hepatic microsomes from phenobarbital, 3-methylcholanthrene and polychlorinated biphenyls pretreated rats

Pretreatment	Phenylethanediols formed (nmoles/mg protein/min)		*S*-(+)-/*R*-(−)-Phenylethanediols
	S-(+)-Form	*R*-(−)-Form	
None	3.41	2.72	1.25
Phenobarbital	8.42	5.98	1.41
3-Methylcholanthrene	5.25	4.15	1.27
Polychlorinated biphenyls	10.35	7.55	1.37

Microsomes (1.0–2.7 mg protein/ml) were incubated with freshly distilled styrene (2 mM) for 60 min at 37° and pH 7.4 in air in the presence of NADP (0.5 mM), glucose 6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 I.U./ml) and $MgCl_2$ (5 mM). Under these conditions, phenyloxirane eantiomers formed from styrene were completely hydrolyzed by microsomal epoxide hydrolase to the corresponding phenylethanediol enantiomers. Phenylethanediols isolated from the incubation mixtures were separated and determined as *R*-(+)-MTPA diesters under the same conditions as described in Fig. 3.

epoxide hydrolase [3, 4], whose activity was almost unchanged during incubation and became rather high relative to the decreasing activities of monooxygenase according to the progress in microsomal lipid peroxidation [33].

After the absence of phenyloxirane in the incubation mixtures was checked at the end of the reaction by GLC, phenylethanediols formed were determined as *R*-(+)-MTPA diesters in the same manner as described earlier. Both *R*-(−)- and *S*-(+)-phenylethanediols were detected in the mixture. Data indicated that only a 13% excess of *S*-(−)-phenyloxirane to the *R*-(+)-oxirane was formed by the monooxygenation of styrene (Table 2). That no further oxidative metabolism occurred with the biologically formed phenylethanediol enantiomers during incubations was confirmed by the quantitative recovery of the separately added enantiomers or racemate from the complete incubation mixtures without styrene.

Liver microsomes from male young adult rats pretreated with phenobarbital, 3-methylcholanthrene or polychlorinated biphenyls enhanced the rate of phenylethanediol formation from styrene when incubated under the afore-mentioned conditons. However, no appreciable alteration was observed in the *R*-(−)- to *S*-(+)- ratio of phenylethanediol enantiomers formed from styrene (Table 2) as well as in the relative rate of the *R*-(−)- to *S*-(+)-diols formed from the corresponding enantiomeric phenyloxiranes as substrates.

DISCUSSION

The present investigation has established stereochemistry in enzymatic glutathione *S*-conjugation, hydrolysis and formation of phenyloxiranes as summarized in Fig. 4. Data indicate that, in rat liver, phenyloxiranes are formed from styrene by microsomal P-450 with a small enantioselectivity and hydrolyzed regiospecifically at significantly different rates by microsomal epoxide hydrolase without inversion of the absolute configuration of their benzylic carbons or conjugated with glutathione highly regioselectively by cytosolic glutathione *S*-transferase, also at significantly different rates.

Both conjugates **1** and **2** enzymatically formed from the *R*-(+)- and *S*-(−)-oxiranes as major products, respectively, could have *S*-benzylic carbons since the conjugation reactions are reasonably assumed to take place in the manner of an S_N2 or S_N2-like mechanism between the epoxide enantiomers and the glutathione sulphydryl group. Similarly, conjugate **1**, enzymatically formed as the minor product from the *S*-(−)-oxirane, and conjugate **2** from the *R*-(+)-isomer, could have *R*-benzylic carbons. The diastereoisomers of each regioisomeric conjugate, however, were inseparable from each other on an octadecysilicone column as well as on a silica column in various solvent systems by HPLC. Purely separated regioisomeric conjugates formed from racemic and enantiomeric phenyloxiranes at alkaline pH were identical with each other by ^{13}C NMR spectrometry. That supported the previous result of ^1H NMR spectroscopic assignment of their structures [24].

The introduction of the glutathione sulphydryl group to the carbon atoms of racemic phenyloxirane was first demonstrated to occur specifically at the benzylic position in an aqueous alkaline medium [14]. Later, the formation of the non-benzylic regioisomer at a smaller ratio to the major benzylic one was reported in the reaction of glutathione with racemic phenyloxirane under similar conditions [17].

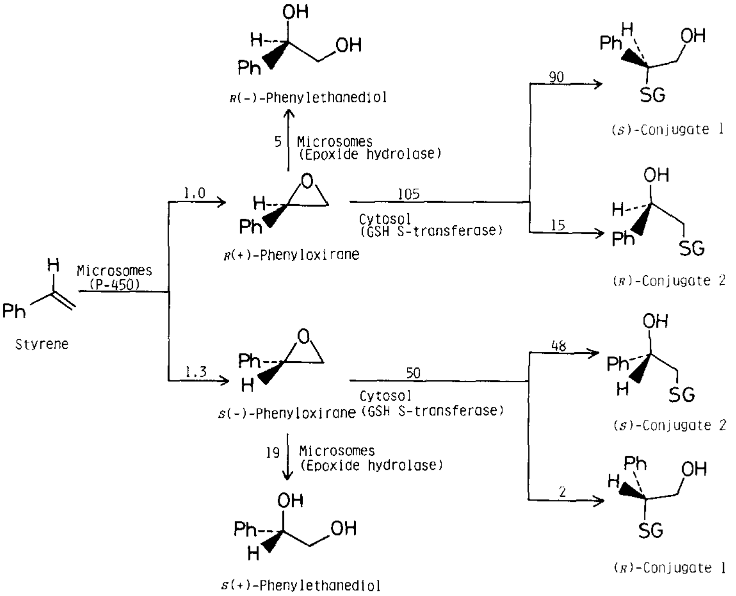


Fig. 4. Stereochemistry in the hepatic metabolism of the vinyl side chain of styrene in the rat. Numerals on the arrows represent relative reaction rates per g tissue (1.0 = 25.4 nmoles/g liver/min), and the chirality symbols, *R* and *S*, for the conjugates **1** and **2** mean the possible absolute configurations of their benzylic carbons.

However, they failed to separate the regioisomers and obtained the conclusion mainly from the result of a treatment of their inseparable conjugate mixture with Raney nickel; phenethyl alcohol and methylphenylcarbinol were the reaction products. As has very recently been demonstrated [24], the Raney nickel desulphuration method is inadequate for the determination of the sulphur-bearing carbon atom in the phenyloxirane–glutathione conjugate molecule since it affords phenyloxirane as a major desulphuration product from both conjugates **1** and **2**. The epoxide formed in the catalytic reaction medium is then hydrogenated to phenethyl alcohol and methylphenylcarbinol. The present study indicated the sulphur atom of glutathione to be introduced to both oxirane carbons (ratio 1:1.4) at benzylic and non-benzylic positions. This fact agrees with the result of the recent work [26] reporting the reaction of racemic phenyloxirane with *N*-acetylcysteine in an alkaline medium, which shows a higher non-benzylic ratio than the case of glutathione.

The enzymatic conjugation of racemic phenyloxirane, however, yielded the benzylic and non-benzylic conjugates (ratio 1.8:1). This result would agree with the previous studies that racemic phenyloxirane given to rats was excreted as benzylic and non-benzylic *N*-acetyl-L-cysteine conjugates of phenylethanols (at a higher ratio of the former to the latter) into their urine [25–27].

The present result of the highly regioselective enzymatic conjugation of *R*-(+)- and *S*-(−)-phenyloxiranes strongly suggests that the regioisomeric ratio observed in the enzymatic conjugation of the racemic epoxide would be a simple reflection of the reactions of glutathione with the chiral substrates at least in the range of zero-order kinetics. All the enzymatic conjugation reactions were carried out by brief incubations in the present investigation in order to decrease the ratio of non-enzymatic products. It is most likely that the observed regioselectivity in the enzymatic glutathione *S*-conjugation of enantiomeric phenyloxirane may be determined by a factor of a chiral–chiral interaction merely between the substrates and glutathione but not necessarily by an enzymatic action. Glutathione *S*-transferase seems only to accelerate the rates of the regioselective conjugations. In the *in vitro* systems used in the present investigation, the rates of enzymatic glutathione conjugation of phenyloxiranes were much faster than those of their enzymatic hydrolysis. To our knowledge, the present investigation provides the first evidence for enzymatic and non-enzymatic regioselective glutathione conjugations of epoxide enantiomers.

Despite the well-recognized importance of glutathione conjugation in the detoxication of epoxides formed from a variety of arenes and olefins [12], structures and homogeneity of these conjugates have not been well studied except in a few cases. Racemic benzo[*a*]pyrene 4,5-oxide [23] and phenyloxirane [24] were the only cases of the separation of enzymatically formed regioisomeric conjugates. Cholesterol α-epoxide, however, has been demonstrated to form a single glutathione conjugate with rat liver glutathione *S*-transferase as a result of the specific introduction of the sulphydryl group to the less hindered 6β-position [22]. The other carbons of the steroid oxirane could not be attacked by the SH from the 6β-side because of the well-known 1,3-diaxial steric hindrance effect of the vicinal 10β- (or C_{19}-) methyl group. Leukotriene A formed from arachidonic acid via its hydroperoxide, 5-HPETE, has also been demonstrated to conjugate with glutathione regiospecifically at the oxirane carbon adjacent to the triene system in murine mast cell tumors [34, 35]. The position of the carbon that bears the sulphur atom in SRS-A (slow reacting substance of anaphylaxis) formed has been assigned after the derivatization to the saturated hydroxy fatty acid by the Raney nickel method [36]. Hydrogenolytic desulphuration by Raney nickel has also been used for the assignment of the sulphur-bearing carbon of the glutathione conjugate and the mercapturic acid from racemic dihydronaphthalene oxide [18, 19].

The MTPA diester method introduced in the HPLC separation of phenylethanediol enantiomers formed from phenyloxirane enantiomers and racemate made it possible to establish the stereochemical course in the enzymatic hydrolysis of these substrates. A further investigation carried out in the present study on the previously observed regioselectivity in the enzymatic hydrolysis of racemic phenyloxirane [16] demonstrated that microsomal hydrolase introduced a hydroxyl group to the non-benzylic oxirane carbon of each enantiomer in a highly regiospecific manner: *R*-(−)-phenylethanediol from *R*-(+)-phenyloxirane and *S*-(+)-phenylethanediol from *S*-(−)-phenyloxirane. There existed a significant difference in V_{max} ($S \gg R$) and K_m ($S \gg R$) between enantiomeric phenyloxiranes when separately used as the microsomal substrates. The microsomal hydrolysis of the enantiomeric epoxides is most likely mediated by a single enzyme, for the *S*-(−)-enantiomer with larger V_{max} and K_m was a poorer substrate than the *R*-(+)-enantiomer when they were simultaneously used as substrates (racemate). This was demonstrated by the preferential formation of *R*-(−)-phenylethandiol rather than the *S*-(+)-diol isomer from the racemic substrate at the earlier stage of the reaction. And then, according to the decrease in concn of *R*-(+)-phenyloxirane which played a role probably as a competitive inhibitor, the rate of phenylethanediol formation from the remaining substrate (about 50%) rapidly increased with a concomitant formation of increasing amounts of the *S*-(+)-diol. A very similar result has recently been reported with racemic, *R*- and *S*-*p*-nitrophenyloxirane without direct determination of the enzymatically formed enantiomeric glycols [37]; in rat liver microsomes, *S*-*p*-nitrophenyloxirane has a larger V_{max} and K_m than the *R*-isomer does. To our knowledge, the present investigation provides the first evidence for the establishment of stereochemistry involving the chiral selectivity which is directly related to the absolute configuration of oxirane carbons in enzymatic hydrolysis of enantiomeric olefin oxides although a variety of stereochemical studies have been made with hepatic microsomal hydrolysis of epoxides such as benzo[*a*]pyrene 4,5- [38], 7,8- [38, 39] and 9,10-epoxides [38], naphthalene epoxide [16], stilbene epoxides [40], mono-substituted stilbene epoxides [41], 9,10-epoxystearates [42], 4-

tert-butyl-1,2-epoxycyclohexanes [43], cholesterol 5,6-epoxides [44], pregnenolone 5,6-epoxides [45] and 2,3-epoxysteroids [46]. Very recent evidence provided by Levin *et al.* [47] for stereochemistry in the microsomal hydrolysis of the enantiomeric arene oxides, 7*S*-(−)- and 7*R*-(+)-benzo[*a*]pyrene 7,8-epoxides, is of interest in view of a similarity in stereoselectivity to the cases of *S*-(−)- and *R*-(+)-phenyloxiranes, for the 7*S*-oxide was hydrolyzed 4 times faster than the 7*R*-isomer by the stereospecific introduction of a hydroxyl group to their C_8.

The small achiral molecule, styrene, was converted to *R*-(−)- and *S*-(+)-phenylethanediols via the corresponding enantiomeric phenyloxiranes by liver microsomes in the presence of an NADPH-generating system (Table 2). Analytical data for the diols were a direct reflection of the stereochemical course of styrene epoxidation by microsomal monooxygenase since under the incubation conditions used it was confirmed that phenyloxiranes formed were completely hydrolyzed to the corresponding phenylethanediols without further metabolism. Unlike their enzymatic hydrolysis, *R*-(−)- and *S*-(+)-phenylethanediols formed from styrene had unexpectedly very similar ratios. The sp^2 carbon side chain of styrene seems to interact sterically in a less restricted manner with the active site of P-450 than the sp^3 carbon side chain of ethyl benzene whose benzyl carbon has been reported by McMahon and Sullivan [48] to be oxidized highly stereoselectively by rat liver to *R*- and *S*-methylphenylcarbinols (ratio 4:1). They also demonstrated that the ratio became smaller when the animal was pretreated with phenobarbital [49]. The microsomal oxidation of the vinyl side chain of styrene was remarkably enhanced by the pretreatment of rats with phenobarbital, 3-methylcholanthrene and polychlorinated biphenyls, but little difference was observed in the *R*-/*S*-ratio. Maylin *et al.* [50], however, demonstrated a small difference later in rates of microsomal conversion of ethyl benzene to the *R*- and *S*-carbinols and pointed out that McMahon *et al.* [48, 49] failed to estimate the preferential conversion of the *S*-isomer by soluble dehydrogenase into acetophenone. Epoxidation of sp^2 carbons of cyclic compounds by microsomal monooxygenase has been demonstrated to proceed in a highly stereoselective manner, e.g. epoxidation of benzo[*a*]pyrene 7,8-carbons [41], benzo[*a*]pyrene 7,8-diol 9,10-carbons [39], and estratetraenol 16,17-carbons [51].

REFERENCES

1. K. C. Leibman and E. Ortiz, *Molec. Pharmac.* **4**, 201 (1968).
2. H. Vainio, R. Pääkkönen, K. Rönnholm, V. Raunio and O. Pelkonen, *Scand. J. Work Envir. Hlth* **3**, 147 (1976).
3. T. Watabe, M. Isobe, T. Sawahata, K. Yoshikawa, S. Yamada and E. Takabatake, *Scand. J. Work Envir. Hlth* **4**, Suppl. 2, 142 (1978).
4. T. Watabe, M. Isobe, K. Yoshikawa and E. Takabatake, *J. Pharm. Dyn.* **1**, 98 (1978).
5. M. O. James, J. R. Fouts and J. R. Bend, *Biochem. Pharmac.* **25**, 187 (1976).
6. F. Oesch, in *Progress in Drug Metabolism* (Eds. J. W. Bridges and L. F. Chasseaud), Vol. 3, p. 253. John Wiley, New York (1979).
7. C. H. Walker, P. Bentley and F. Oesch, *Biochim. biophys. Acta* **539**, 427 (1978).
8. T. Hayakawa, R. A. Lemahieu and S. Udenfriend, *Archs Biochem. Biophys.* **162**, 223 (1974).
9. T. Hayakawa, S. Udenfriend, H. Yagi and D. M. Jerina, *Archs Biochem. Biophys.* **170**, 438 (1975).
10. J. R. Bend, B. R. Smith, L. M. Ball and H. Mukhtar, in *Conjugation Reactions in Drug Biotransformation* (Ed. A. Aitio), p. 3. North-Holland, Biochemical Press, Amsterdam (1978).
11. F. Oesch, *Xenobiotica* **3**, 305 (1972).
12. D. M. Jerina and J. R. Bend, in *Biological Reactive Intermediates* (Eds. D. J. Jollow, J. J. Kocsis, R. Snyder and H. Vainio), p. 207. Plenum Press, New York (1979).
13. K. C. Leibman, *Envir. Hlth Perspect.* **11**, 115 (1975).
14. A. J. Ryan and J. R. Bend, *Drug. Metab. Dispos.* **5**, 363 (1977).
15. K. Yoshikawa, M. Isobe, T. Watabe and E. Takabatake, *Mutation Res.* **78**, 219 (1980).
16. D. M. Jerina, H. Ziffer and J. W. Daly, *J. Am. chem. Soc.* **92**, 1056 (1970).
17. J. Pachecka, P. Gariboldi, L. Cantoni, G. Belvedere, E. Mussini and M. Salmona, *Chem. Biol. Interact.* **27**, 313 (1979).
18. J. Booth, E. Boyland, T. Sato and P. Sims, *Biochem. J.* **77**, 182 (1960).
19. E. Boyland and P. Sims, *Biochem. J.* **77**, 175 (1960).
20. D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg and S. Udenfriend, *Biochemistry* **9**, 147 (1970).
21. A. M. Jeffery and D. M. Jerina, *J. Am. chem. Soc.* **97**, 4427 (1975).
22. T. Watabe, T. Sawahata and J. Horie, *Biochem. biophys. Res. Commun.* **87**, 469 (1979).
23. O. Hernandez, M. Walker, R. H. Cox, G. L. Foureman, B. R. Smith and J. R. Bend, *Biochem. biophys. Res. Commun.* **96**, 1494 (1980).
24. T. Watabe, A. Hiratsuka, N. Ozawa and M. Isobe, *Biochem. Pharmac.* **30**, 390 (1981).
25. F. Seutter-Berlage, L. P. C. Delbressine, F. L. M. Smeets and H. C. J. Ketelaars, *Xenobiotica* **8**, 413 (1978).
26. L. P. C. Delbressine, P. J. VanBladeren, F. L. M. Smeets and F. Seutter-Berlage, *Xenobiotica* **11**, 589 (1981).
27. T. Watabe, N. Ozawa and K. Yoshikawa, *J. Pharm. Dyn.* **5**, 129 (1982).
28. T. Watabe, N. Ozawa and K. Yoshikawa, *Biochem. Pharmac.* **30**, 1695 (1981).
29. E. L. Eliel and D. W. Delmonte, *J. org. Chem.* **21**, 596 (1956).
30. J. A. Dale and H. S. Mosher, *J. org. Chem.* **35**, 4002 (1970).
31. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
32. T. Watabe, M. Isobe, K. Yoshikawa and E. Takabatake, *J. Pharm. Dyn.* **1**, 301 (1978).
33. T. Watabe and K. Akamatsu, *Biochem. Pharmac.* **23**, 1079 (1974).
34. R. C. Murphy, S. Hammarström and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **76**, 4275 (1979).
35. S. Hammarström, B. Samuelsson, D. A. Clark, G. Goto, A. Marfat, C. Mioskowski and E. J. Corey, *Biochem. biophys. Res. Commun.* **92**, 946 (1980).
36. M. Rosenberger and C. Neukom, *J. Am. chem. Soc.* **102**, 5426 (1980).
37. R. B. Westkaemper and R. P. Hanzlik, *Archs Biochem. Biophys.* **208**, 195 (1981).
38. D. R. Thakker, H. Yagi, W. Levin, A. Y. H. Lu, A. H. Conney and D. M. Jerina, *J. biol. Chem.* **252**, 6328 (1977).
39. S. K. Yang, D. W. McCourt, P. P. Roller and H. V.

Gelboin, *Proc. natn. Acad. Sci. U.S.A.* **73**, 2594 (1976).
40. T. Watabe, K. Akamatsu and K. Kiyonaga, *Biochem. biophys. Res. Commun.* **44**, 199 (1971).
41. P. M. Dansette, V. B. Makedonska and D. M. Jerina, *Archs Biochem. Biophys.* **187**, 290 (1978).
42. T. Watabe and K. Akamatsu, *Biochim. biophys. Acta* **279**, 297 (1972).
43. G. Bellucci, G. Berti, G. Ingrosso and E. Mastrorilli, *J. org. Chem.* **45**, 299 (1980).
44. T. Watabe, M. Kanai, M. Isobe and N. Ozawa, *Biochim. biophys. Acta* **619**, 414 (1980).
45. T. Watabe, M. Kanai, M. Isobe and N. Ozawa, *Biochem. biophys. Res. Commun.* **92**, 977 (1980).
46. T. Watabe, K. Kiyonaga, K. Akamatsu and S. Hara, *Biochem. biophys. Res. Commun.* **43**, 1252 (1971).
47. W. Levin, M. K. Buening, A. W. Wood, R. L. Chang, B. Kedzierski, D. R. Thakker, D. R. Boyd, G. S. Gadaginamath, R. N. Armstrong, H. Yagi, J. M. Karle, T. J. Slaga, D. M. Jerina and A. H. Conney, *J. biol. Chem.* **255**, 9067 (1980).
48. R. E. McMahon and H. R. Sullivan, *Life Sci.* **5**, 921 (1966).
49. R. E. McMahon, H. R. Sullivan, J. C. Graig and W. E. Pereira, *Archs Biochem. Biophys.* **132**, 575 (1969).
50. G. A. Maylin, M. J. Cooper and M. W. Anders, *J. med. Chem.* **16**, 606 (1973).
51. T. Watabe, S. Ichihara and T. Sawahata, *J. biol. Chem.* **254**, 10,720 (1979).