BBA 66257

A RADIOMETRIC ASSAY FOR HEPATIC EPOXIDE HYDRASE ACTIVITY WITH [7-^{3}H] STYRENE OXIDE*

F. OESCH, D. M. JERINA AND J. DALY

Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

(Received September 7th, 1970)

SUMMARY

[7-^{3}H]Styrene oxide is converted to [7-^{3}H]styrene glycol by a hepatic epoxide hydrase. Differential extraction of the incubation medium provides the basis for a rapid, simple method to assay epoxide hydrase activity. Unreacted substrate is first removed by extraction into petroleum ether followed by extraction of the glycol into ethyl acetate for assay by scintillation spectrometry. Enzyme activity is present in liver and kidneys and is localized exclusively in the microsomal fraction. The simplicity of the present assay permits the use of epoxide hydrase a a marker enzyme for microsomal membranes. The specific activity of this enzyme in hepatic microsomes increases during maturation of rats and following pretreatment of rats with phenobarbital or 3-methylcholanthrene.

INTRODUCTION

Enzymatic hydration of epoxides is now recognized as an important metabolic step in the detoxification of foreign compounds by higher organisms and is particularly important to the metabolism of aromatic substrates where arene oxides, such as 1,2-naphthalene oxide have been identified as key intermediates[1]. The epoxide hydrase present in liver homogenates converts a variety of epoxides, including cyclohexene oxide[2], indene oxide[2,3], octene oxide[4], chlordene oxide[5], dieldrin[6], 1,2-naphthalene oxide[1], benzene oxide[2], 8,9-indane oxide[7], 9,10-phenanthrene oxide[8], and steroidal epoxides[9] to diols. To purify and investigate the properties of this enzyme or enzymes, a convenient assay has been developed with [7-^{3}H]styrene oxide as substrate. The product, [7-^{3}H]styrene glycol, can be readily separated from substrate for assay by differential solvent extraction of the incubation medium.

METHODS

Preparation of [7-^{3}H]styrene oxide

A solution of *m*-chloroperoxybenzoic acid (4.16 g, 85% technical grade, Al-

* A preliminary report of this work has appeared: *Federation Proc.*, 29 (1970) 473.

drich Chemical Co.) in methylene chloride (57 ml) was added slowly to [7-^{3}H]styrene (2 g, Tracer Lab., Watham, Mass., previously diluted to a specific activity of 0.372 μC/μmole with styrene, Eastman Kodak) in methylene chloride (28 ml) while stirring at 0°. Stirring was continued at 0° for an additional 7 h. The solution was filtered, washed once with 30 ml 10% aqueous NaOH and then twice with water, dried over Na_2SO_4 and evaporated *in vacuo*. Distillation of the resulting oil (b.p. 88°, 26 mm) provided 1.66 g (72%) of styrene oxide as confirmed by NMR spectroscopy, thin-layer chromatography and color reaction (epoxide reagent, 10 mg methyl red and 5 gNaI per 100 ml isopropanol, heat 5 min at 100°). Radiochemical purity was demonstrated by thin-layer chromatography on silica gel GF plates with authentic styrene oxide in four different solvent systems (benzene–chloroform (1:1, by vol.); benzene–light petroleum (1:1, by vol.); chloroform–light petroleum (1:1, by vol.); benzene–ethyl acetate–chloroform (1:1:1, by vol.). After dilution with freshly distilled styrene oxide to a specific activity of 35.4 μC/mmole, the product was stored at −15°.

Assay of epoxide hydrase activity with [^{3}H]styrene oxide

Incubation mixtures were prepared at 0° by sequential addition of 0.1 ml 0.5 M Tris buffer (pH 9) containing 0.1% (w/w) Tween 80, water to make the final volume 0.4 ml, 0.02 ml acetonitrile containing 0.8 μmole [^{3}H]styrene oxide (62 800 disint./min), and 0.02–0.2 ml of enzyme preparation. After incubation at 37° for 5 min, the reaction was terminated by the extraction of the substrate into 10 ml. of light petroleum (b.p. 30–60°). Removal of the organic phase was readily accomplished by freezing the aqueous phase in dry ice–acetone and decanting the light petroleum. After thawing, the aqueous phase was again extracted with 10 ml. of petroleum ether. The product, [^{3}H]styrene glycol was then extracted from the aqueous phase into 2 ml. of ethyl acetate. An aliquot (0.2 ml) was counted in Bray's phosphor solution with a counting efficiency of 37.2 ± 0.5%. Boiled enzyme preparations (15 min, 100°) served as controls.

Preparation of homogenates and subcellular fractions

Chilled tissue was cut into small pieces and homogenized in 3 vol. of cold 1.19% KCl or 0.25 M sucrose. Liver homogenates were fractionated by centrifugation at 600 × *g* for 15 min to remove cell debris and nuclei, at either 8500 or 20 000 × *g* for 15 min to obtain mitochondria and then at 100 000 × *g* for 1 h to obtain microsomes. Nitrogen in the preparations was measured by the Kjeldahl method. Sprague–Dawley rats (200–300 g), Hartley guinea pigs (250–300 g), New Zealand white rabbits (2.5–3 kg), and a Rhesus monkey (5 kg) were used.

RESULTS AND DISCUSSION

Assay for epoxide hydrase activity

Several methods for measuring epoxide hydrase activity were considered. Colorimetric assays based on *vic*-glycol formation with 1,2-naphthalene oxide[1] or styrene oxide[10] as substrate proved too time consuming for routine assay during enzyme purification. A colorimetric assay based on the formation of colored complexes of styrene glycol with potassium triacetylosmiate[11] was insensitive and did not

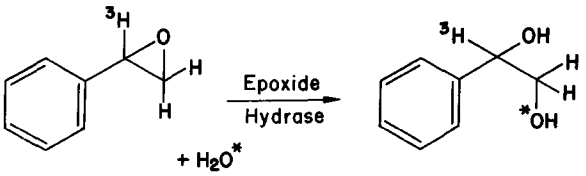

Fig. 1. Enzymatic conversion of [7-^{3}H]styrene oxide to [7-^{3}H]styrene glycol. *Enzymatic attack by water occurs exclusively at the 8-position[8].

follow Beer's law. Subsequently, a radiometric assay based on the conversion of [7-^{3}H]styrene oxide to [7-^{3}H]styrene glycol (Fig. 1) was developed. Substrate was quantitatively extracted into light petroleum. Thus, only 0.13% of substrate remained in the incubation medium after two extractions while only 8.8% of the product, [7-^{3}H]styrene glycol was removed. The remaining product was then extracted into ethyl acetate for assay by liquid scintillation spectrometry. Recovery of enzymatically synthesized [7-^{3}H]styrene glycol was 86 $\pm$ 1.7% through the entire assay

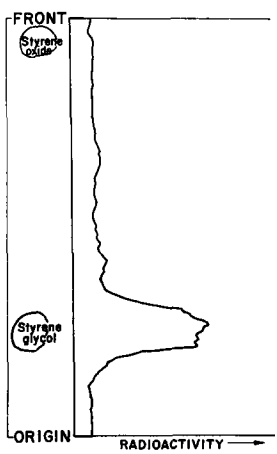

Fig. 2. Thin-layer chromatoplate of the radioactive product which extracts into ethyl acetate after incubation of [7-^{3}H]styrene oxide with guinea pig liver microsomes. Styrene glycol and styrene oxide added as standards. Chromatography on a silica gel GF plate (Analtech Co.) with chloroform–ethyl acetate (1:1, by vol.). Plate scanned for radioactivity in a Vanguard thin-layer chromatoplate scanner. Similar results were obtained in two other solvent systems.

procedure. Assay values presented here have not been corrected for recovery. Incubations at zero time and with boiled preparations served as controls and assayed 640–700 disint./min per incubation above background. Preparations of microsomes heated 15 min at 100° were completely inactive, while 5 min at 60° caused no loss of activity. At 0° the enzyme catalyzed no detectable formation of product during 10 min. Incubations for 5 min with various enzyme preparations under the standard conditions assayed 6000–24 000 disint./min per incubation (10–40% conversion) after correction for controls. Radioactivity extracted into ethyl acetate was exclusively associated with carrier styrene glycol as shown by thin-layer chromatography (Fig. 2). A concentration of [7-^{3}H]styrene oxide ($2 \cdot 10^{-3}$ M) sufficient to

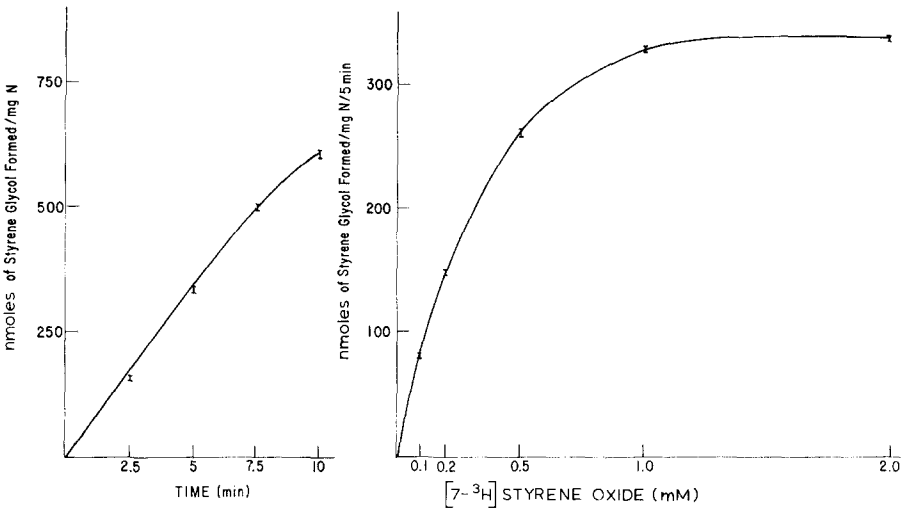

Fig. 3. Enzymatic hydration of [7-^{3}H]styrene oxide as function of time and substrate concentration. Microsomes were prepared by centrifugation of the 8500 × *g* supernatant of guinea pig liver homogenates (isotonic sucrose) at 100 000 × *g* for 1 h and incubated under the standard conditions with [7-^{3}H]styrene oxide. Each incubation (0.4 ml) contained 0.48 mg nitrogen. Vertical bars represent values of duplicate samples.

saturate the enzyme was employed in the assay (Fig. 3). The time-course of product formation was linear for >7.5 min with microsomal preparations (Fig. 3).

Distribution of epoxide hydrase activity

Activity was associated with "mitochondrial" and microsomal fractions after differential centrifugation of rat and rabbit liver homogenates prepared with 3 vol. of isotonic KCl using a glass homogenizer with a Teflon pestle (Table I). Purity of mitochondrial fraction was determined by assaying a microsomal marker enzyme, glucose-6-phosphatase[12]. Glucose-6-phosphatase activity was high in both microsomal and "mitochondrial" fractions when homogenization was carried out in isotonic KCl.

TABLE I

EPOXIDE HYDRASE ACTIVITY IN SUBCELLULAR FRACTIONS OF LIVER HOMOGENATES PREPARED WITH ISOTONIC KCl

Fractions: (1) liver homogenized with 3 vol. of isotonic KCl; (2) prepared from 600 × *g* supernatant by centrifuging at 20 000 × *g* for 15 min, washing, and resuspending in isotonic KCl; (3) prepared from 20 000 × *g* supernatant by centrifuging at 100 000 × *g* for 1 h, washing and resuspending in isotonic KCl; (4) 100 000 × *g* supernatant.

Fraction	*nmoles of styrene glycol per mg N per 5 min*	
	Rat	*Rabbit*
(1) Homogenate	43	55
(2) "Mitochondrial" fraction	112	128
(3) Microsomal fraction	138	168
(4) Soluble fraction	13	24

TABLE II

EPOXIDE HYDRASE AND GLUCOSE-6-PHOSPHATASE ACTIVITY IN PARTICULATE FRACTIONS FROM MALE GUINEA PIG LIVER HOMOGENATES PREPARED IN ISOTONIC KCl OR SUCROSE

Glucose-6-phosphatase activity was assayed according to SWANSON[15] and the liberated phosphate determined by the method of LOWRY[16]. Epoxide hydrase activity was determined with [7-^{3}H]-styrene oxide under the standard conditions. Fractions: (1) prepared from 600 × *g* supernatant by centrifuging at 8500 × *g* for 15 min, washing and resuspending in isotonic medium; (2) prepared from 8500 × *g* supernatant by centrifuging at 100 000 × *g* for 1 h, washing and resuspending in isotonic medium.

Fraction	*Specific activity (nmoles product per mg N per 5 min*			
	Homogenate in isotonic KCl		*Homogenate in isotonic sucrose*	
	Glucose-6--phosphatase	*Epoxide hydrase*	*Glucose-6--phosphatase*	*Epoxide hydrase*
(1) Mitochondrial	244	263	38	42
(2) Microsomal	266	388	245	392

In contrast, when isotonic sucrose was employed, both glucose-6-phosphatase and epoxide hydrase activity were found exclusively in the microsomal fraction (Table II). Because of the simplicity of the present assay, epoxide hydrase might also be used as a marker enzyme for microsomal membranes.

Epoxide hydrase activity was found in liver and kidney of rat and guinea pig, but was not detected in six other tissues (Table III). The specific activity of epoxide hydrase in liver microsomes varied between species (Fig. 4), but not between sexes in rat, guinea pig and rabbit. The specific activity of epoxide hydrase was highest in monkey liver.

Induction of epoxide hydrase activity and increase during maturation of rats

The specific activity of epoxide hydrase in liver microsomes was increased 300% by pretreatment of male rats with phenobarbital and 150% by pretreatment with 3-methylcholanthrene (Fig. 4). Significant enhancement of epoxide hydrase

TABLE III

EPOXIDE HYDRASE ACTIVITY IN VARIOUS ORGANS

*Tissue***	*nmoles of styrene glycol per 5 min**			
	*Female rat****		*Male guinea pig*‡*	
	Per mg N	*Per organ*	*Per mg N*	*Per organ*
Liver	138	15 500	175	39 400
Kidney	21	92	13	178

* Incubated with [7-^{3}H]styrene oxide under the standard conditions.

** No activity was detected in spleen, brain, intestine, lung, heart or muscle.

*** Microsomes prepared from 6000 × *g* supernatant (10 min, isotonic KCl) by centrifuging at 100 000 × *g* for 1 h, washing and resuspending in isotonic KCl.

‡ 8500 × *g* supernatant (isotonic sucrose).

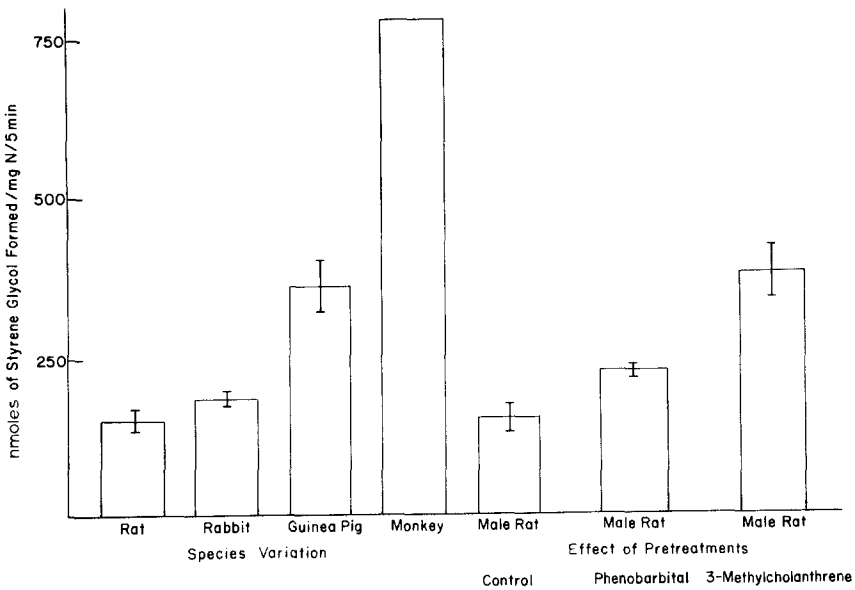

Fig. 4. Epoxide hydrase activities in liver microsomes from various species and effect of pretreatment of male rats with phenobarbital or 3-methylcholanthrene. Microsomes were prepared by centrifugation of the 8500 × *g* supernatant (isotonic KCl) at 100 000 × *g* for 1 h and incubated under the standard conditions with [7-^{3}H]styrene oxide. Species values ± S.D. are means from 4 male and 4 female rats, 3 male and 3 female rabbits, 4 male and 4 female guinea pigs and 1 male rhesus monkey. No sex differences were noted. For induction experiments, male rats were treated for 3 days with either 3-methylcholanthrene (40 mg/kg in cottonseed oil, one injection/day) or with 0.2% sodium phenobarbital in drinking water and sacrificed on day 4. Microsomes were prepared by centrifugation of the 20 000 × *g* supernatant (isotonic KCl) at 100 000 × *g* for 1 h and incubated under the standard conditions. Values ± S.D. are means from 8 rats. Enzyme activity was not increased by pretreatment of guinea pigs or rabbits with phenobarbital or 3-methylcholanthrene.

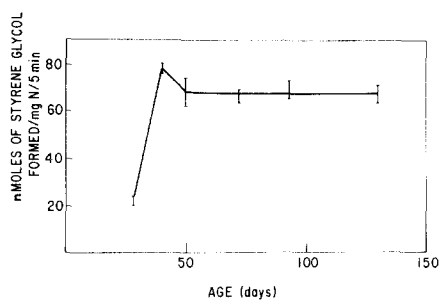

Fig. 5. Epoxide hydrase activity in rat liver microsomal preparation as a function of the age of male rats. Rats at 28 days weighed 49–53 g; at 40 days, 102–120 g; at 50 days, 155–170 g; at 72 days, 220–240 g; at 93 days, 255–285 g; and at 130 days, 340–360 g. Values ± S.D. are means from 4 rats. The 20 000 × *g* supernatant (isotonic KCl) was incubated with [7-^{3}H]styrene oxide under the standard conditions.

activity was not evoked by pretreatment of rabbits or guinea pigs with these compounds. No sex differences in response to pretreatment were noted in these species. The specific activity of epoxide hydrase in liver microsomes increased nearly 4-fold during maturation of male rats from 28 to 40 days of age (Fig. 5).

CONCLUSIONS

These studies demonstrate the presence of an epoxide hydrase in microsomes from rat, rabbit, guinea pig and monkey capable of catalyzing the conversion of styrene oxide to styrene glycol. The increase in specific activity of this microsomal enzyme during maturation of rats and after pretreatment with phenobarbital and 3-methylcholanthrene parallels similar increases in other drug metabolizing enzymes, *i.e.* microsomal oxygenases[13,14]. Utility of this radiometric assay has been demonstrated during 40-fold purification of the enzyme from guinea pig liver homogenates[17] and in examination of the effects of cosubstrates and inhibitors on the enzyme[18]. The purified enzyme also catalyzes the hydration of a variety of other epoxides[19].

ACKNOWLEDGMENTS

One of the authors (F.O.) is a Fellow of the Schweizerische Stiftung für Stipendien auf dem Gebiete der Chemie, 1969–1970 and Fellow in the Visiting Program of the U.S. Public Health Service, 1970–present.

REFERENCES

1 D. M. JERINA, J. W. DALY, B. WITKOP, P. ZALTZMAN-NIRENBERG AND S. UDENFRIEND, *Biochemistry*, 9 (1969) 147.
2 D. JERINA, J. DALY, B. WITKOP, P. ZALTZMAN-NIRENBERG AND S. UDENFRIEND, *Arch. Biochem. Biophys.*, 128 (1968) 176.
3 K. C. LEIBMAN AND E. ORTIZ, *Mol. Pharmacol.*, 4 (1968) 201.
4 T. WATABE AND E. W. MAYNERT, *Pharmacologist*, 10 (1968) 202.
5 G. T. BROOKS, S. E. LEWIS AND A. HARRISON, *Nature*, 220 (1968) 1034.
6 G. T. BROOKS, A. HARRISON AND S. E. LEWIS, *Biochem. Pharmacol.*, 19 (1970) 255.
7 J. W. DALY, D. M. JERINA, H. ZIFFER, B. WITKOP, F. G. KLARNER AND E. VOGEL, *J. Am. Chem. Soc.*, 92 (1970) 702.
8 D. M. JERINA, H. ZIFFER AND J. W. DALY, *J. Am. Chem. Soc.*, 92 (1970) 1056.
9 H. BREUER AND R. KNUPPEN, *Biochim. Biophys. Acta*, 49 (1961) 620.
10 K. C. LEIBMAN AND E. ORTIZ, *Anal. Chem.*, 40 (1968) 251.
11 R. CRIEGE, B. MARCHAND AND H. WANNOWIUS, *Analen Chem.*, 550 (1942) 99.
12 H. G. HERS AND C. DE DUVE, *Bull. Soc. Chim. Biol.*, 32 (1950) 20.
13 T. E. GRAM, A. M. GUARINO, D. H. SCHROEDER AND J. R. GILLETTE, *Biochem. J.*, 113 (1969) 681.
14 A. H. CONNEY, *Pharmacol. Rev.*, 19 (1967) 317.
15 M. A. SWANSON, *J. Biol. Chem.*, 184 (1950) 647.
16 O. H. LOWRY, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 4, Academic Press, New York, 1957, p. 366.
17 F. OESCH AND J. W. DALY, *Biochim. Biophys. Acta*, 227 (1971) 692.
18 F. OESCH, N. KAUBISCH, D. M. JERINA anff J. W. DALY, in preparation.
19 F. OESCH, D. M. JERINA AND J. W. DALY, *Arch. Biochem. Biophys.*, submitted.