ANALYTICAL BIOCHEMISTRY **106**, 476–485 (1980)

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# A Rapid Radiometric Assay for Mammalian Cytosolic Epoxide Hydrolase

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Received January 11, 1980

A rapid and sensitive radiometric assay for cytosolic epoxide hydrolase is described. The assay is based on the highly efficient partitioning of unreacted 1-phenyl-1,2-epoxybutane ($\beta$-ethylstyrene oxide) from an aqueous mix into isooctane, with the product diol being retained in the aqueous phase. The *trans*-epoxide is an excellent substrate for both the glutathione transferase and epoxide hydrolase present in mouse liver cytosol, and both enzymes may be monitored simultaneously after appropriate modifications of the assay are made. Also, *trans*-$\beta$-ethylstyrene oxide is hydrated much faster than the *cis*-isomer in the hepatic cytosol of three different mammalian species, and both isomers are poor substrates for the microsomal epoxide hydrolase. Mouse liver cytosol hydrates the *trans*-epoxide at 69 nmol/min-mg protein, and a $K_m$ of $7.2 \times 10^{-5}$ M is observed. Reduction of 1-phenyl-1-bromo-2-butanone with sodium borotritide and subsequent ring closure of the bromohydrin mix with base was a highly efficient route for radiolabeling $\beta$-ethylstyrene oxide. The resulting *cis*- and *trans*-epoxides were separable by semipreparative high-performance liquid chromatography.

In the past two decades, epoxide hydrolases (EC 3.3.2.3, formerly EC 4.2.1.63) have been studied with increasing fervor largely because of the toxicological importance of many of the epoxide substrates on which they act (1–4). Epoxides are known to be carcinogens, teratogens, and mutagens in mammals. Man's inadvertent exposure to a host of environmentally introduced (5–8) as well as endogenously formed (9,10) epoxides has led to considerable effort in establishing the role of epoxide hydrolase in the detoxification or, in some cases, the toxification (11) of such compounds. However, investigators have mostly focused on microsomal epoxide hydrolases and glutathione transferases but overlooked the possible role extramicrosomal epoxide hydrolases might have in the disposing of potentially hazardous epoxides in mammalian systems.

Recently, this laboratory has established cytosolic epoxide hydration as a route of epoxide degradation in mammals (12–16). The tedium involved in assaying epoxide hydrolase by available methods together with the unfortuitous selection of inappropriate substrates for the cytosolic enzyme when classical subcellular distribution studies were performed had previously led to the cytosolic epoxide hydrolase being overlooked. Thus, the development of a rapid assay that will distinguish the cytosolic from the microsomal epoxide hydrolase would be of great utility.

The most cited assays for microsomal epoxide hydrolase (17–21) are radiometric assays utilizing differential solvent extraction, tlc,[1] and/or other purification or extraction steps prior to lsc. Similarly, radiometric assays requiring tlc analysis of methyl

[1] Abbreviations used: glc, gas–liquid chromatography; hplc, high-performance liquid chromatography; *I*, ionic strength; JH, juvenile hormone; lsc, liquid scintillation counting; SD, standard deviation; tlc, thin-layer chromatography; *t*, *trans*; *c*, *cis*.

0003-2697/80/120476-10$02.00/0



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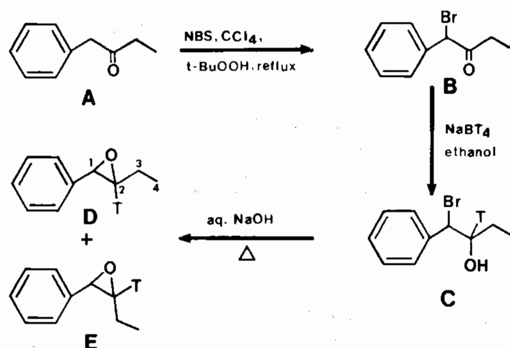


FIG. 1. Scheme for radiolabeling β-ethylstyrene oxide. The geometrical isomers of the epoxide were separated by hplc.

epoxystearates (13), juvenile hormone, and various juvenoid epoxides (22,23) have fostered much of the work on cytosolic epoxide hydrolase. These assays are laborious and thus a partition assay for epoxide hydrolase activity on JH mimics was developed (24). These terpenoid epoxides, however, are awkward models for many of the xenobiotic epoxides mammals encounter and, also, may undergo alternative routes of metabolism. Recently, substrate specificity studies on the cytosolic epoxide hydrolase ((14,16), L. Hasegawa *et al.*, unpublished data) have identified simple epoxides which are excellent substrates for cytosolic epoxide hydrolase but are only slowly converted by the microsomal enzyme. One of these substrates, *trans*-β-ethylstyrene oxide (*trans*-1-phenyl-1,2-epoxybutane), has highly favorable characteristics for a partition assay. The remainder of this report will describe the radiolabeling of this compound and its use in the development of a rapid partition assay for mammalian cytosolic epoxide hydrolase.

## METHODS

### *Synthesis and Separation of trans- and cis-β-[2-$^3$H]Ethylstyrene Oxide*

A Varian EM-390 was resorted to for all NMR studies while a Bausch & Lomb Spectronic 21 was used for uv-visible spectrophotometry. All compounds were analyzed at least by tlc (EM Reagents, silica gel F-254, 250 μm) with visualization by quenching of gel fluorescence, 4-(*p*-nitrobenzyl)pyridine (selective for alkylating agents), or lead tetraacetate (selective for diols) (22).

To prepare 1-bromo-1-phenyl-2-butanone (**B**, Fig. 1), a solution of 1-phenyl-2-butanone (**A**) (25 mmol, Aldrich) in 100 ml carbon tetrachloride was refluxed 16 h with *N*-bromosuccinimide (30 mmol, MC & B) and 100 μl t-butyl hydroperoxide to give the desired product (92% yield by NMR) which upon distillation ($bp_{0.03}$ 66–67°C) yielded a viscous yellow oil (solid at −20°C); NMR ($CCl_4$) δ 1.05 (3H, t, $J$ = 7.4 Hz), 2.54 (2H, qd, $J$ = 7.4 Hz), 5.32 (1H, s), 7.34 (5H, m). The product was 98% pure by NMR, tlc, and glc but after long-term storage (e.g., 5 weeks at −20°C) was chromatographed on silica gel layers (hexane–ether) immediately prior to use.

To 99 mg of 1-bromo-1-phenyl-2-butanone (**B**) was added 300 μg of sodium borohydride (J. T. Baker) in a total volume of 0.6 ml of absolute ethanol and following stirring at ice temperature for 10 min the entire mixture was transferred with 0.5 ml ethanol to a bottle containing 3.9 mg (25 mCi) of sodium borotritide (New England Nuclear, 240 mCi/mmol). The mixture was stirred 30 min at ice temperature, then 15 min at room temperature, 5 mg of sodium borohydride added in 0.5 ml ethanol, and then stirred an additional 20 min. The resulting bromohydrin (**C**) mixture was treated with aqueous sodium hydroxide (3 $N$, 0.3 ml) at room temperature for 20 min and then at 50°C for 20 min, diluted with 1.0 ml water, and extracted (3×) with 1.0 ml of 20% ether in hexane. The organic phase was washed with 1.5 ml saturated aqueous sodium chloride, concentrated to 0.4 ml under nitrogen, and applied to a hexane equilibrated 0.8 × 8-cm column of ether-washed silica gel (Mallinckrodt, 100 mesh) with a 1.5-cm plug of anhydrous potassium carbonate at the load-

ing end. The sample was washed into the column with 2 ml hexane and the desired epoxide mix successfully eluted with 9 ml of 20% ether in hexane. Following concentration under nitrogen, the mixture of *trans*- and *cis*-β-ethylstyrene oxides (**D,E**) ($t/c$ = 44/56 by glc) were separated by hplc on a tapered inlet column (1.0 cm i.d. × 50 cm) of 10-μm silica gel (Spherosorb, Specta-Physics) with 1.5% ether in hexane at 3.2 ml/min (retention times in min: *t*, 19.4; *c*, 20.7). Fractions were collected at 20-s intervals and their isomer content monitored by flame-ionization detection on a Hewlett–Packard 5710A glc (2.5% OV-101, Gas Chrom Q 100–200 mesh, 1.5-mm × 1.2-m glass column, 87°C, $N_2$ 20 ml/min). Baseline separation of isomers (retention times: *c*, 6.2 min; *t*, 7.3 min) was achieved. Fractions of $99^+$% isomer purity by peak area (Hewlett–Packard 3380A integrator) were combined, and the remaining isomer-rich fractions rechromatographed by hplc until $99^+$% geometrical purity was achieved. An overall yield of radioactivity of greater than 95% and a specific activity of 52 mCi/mmol was obtained ($\epsilon_{256}$ = 163 $M^{-1} cm^{-1}$ for *trans*-epoxide). Chemical purity (>98%), radiopurity (>98%), and geometrical purity (>99%) of the radiolabeled β-ethylstyrene oxides were further confirmed by glc, tlc, and analytical hplc on a 0.2 × 125-cm Spherosorb (5 μm, packed in 25-cm segments joined by drilled-out ¼-in. to ¼-in. Swagelok unions) column which provided baseline separation of the epoxides (retention times: *t*, 24.6 min; *c*, 26.3 min; 2% ether in hexane). Samples were stored in sealed glass ampules at −20°C until required.

### *Synthesis of Epoxides and Standard Diols for the Partition Assay*

Cold *cis*-β-ethylstyrene oxide (**E**) was synthesized and purified by a similar route as above; NMR ($CCl_4$) lit. (25) δ 0.85 (3H, t, $J$ = 7.5 Hz), 1.21 (2H, m), 2.97 (1H, td, $J$ = 6.2, 4.2 Hz), 3.88 (1H, d, $J$ = 4.2 Hz), 7.24 (5H, s). *trans*-β-Ethylstyrene oxide (**D**) was synthesized as above or from *trans*-1-phenyl-1-butene (Chemical Samples Co., Columbus, Ohio) either via the bromohydrin generated with *N*-bromosuccinimide (26) or by *m*-chloroperbenzoic acid oxidation. Both methods gave an isomer purity of at least 95% *trans*-epoxide by NMR ($CCl_4$) lit. (25): δ 1.04 (3H, t, $J$ = 7.6 Hz), 1.63 (2H, qd, $J$ = 6.7 Hz), 2.72 (1H, td, $J$ = 5.4, 1.9 Hz), 3.42 (1H, d, $J$ = 1.9 Hz), 7.20 (5H, s).

Radiolabeled β-ethylstyrene glycol was synthesized by incubation of the *trans*-[$^3$H]epoxide in 0.5 M sodium acetate buffer (pH 4) with 0.5% ethanol at room temperature for 2.7 days in the dark (24), then purified by tlc on silica gel (benzene–propanol, 20:1) relative to authentic diol standards. Radiopurity (>98%) was confirmed by cochromatography in two different solvent systems and by subsequent radioautography. β-Ethylstyrene glycol standards were prepared either by acid hydration (27) of the *trans*-epoxide to give an *erythro*/*threo* mix of 7/3 (NMR) or by mild acid hydration in sodium acetate buffer at 70°C upon which an *erythro*/*threo* mix of 3/7 was obtained; NMR ($CDCl_3$) lit. (28) benzylic proton *threo* (δ 4.37, $J$ = 7.0 Hz) and *erythro* (δ 4.61, $J$ = 4.5 Hz). The cyclic *erythro*- and *threo*-*n*-butylboronates were not readily resolved by glc (2.5% OV-101).

### *Partition Assay for β-Ethylstyrene Oxide Hydrolase*

The assay consisted of preincubating enzyme in Tris–HCl buffer ($I$ = 0.2), pH 7.4, containing 0.1 mM EDTA (total volume, 50 μl) at 37°C for 1 min in a 6 × 50-mm culture tube and then initiating the reaction with radiolabeled plus cold *cis*- or *trans*-β-ethylstyrene oxide introduced in 1 μl ethanol through a Hamilton repeating dispenser such that a final substrate concentration of 5 × $10^{-4}$ M is obtained. Following incubation for 10 min at 37°C, the reaction is terminated by vortexing the incubation mix

with 100 μl of isooctane (Repipet dispenser, Labindustries) for 20 s and then storing the samples on ice. Infrequently, samples were centrifuged at 1000 − 2000$g$ for 5 min to break emulsions. Aliquots of the aqueous phase (20 μl) and organic phase (25 μl) were removed with a Hamilton syringe and analyzed by lsc in a cocktail consisting of OCS (a xylene-based scintillation cocktail, Amersham Corporation) and Triton X-100 (3:1). Efficiency was calculated to be 20% for $^3$H in Beckman minivials. Hydration rates were calculated relative to boiled enzyme controls compensating for the recovery of diol through the assay of 92%. Following the optimization of the partition assay, hydration rates were measured simply by monitoring the aqueous phase.

### *Other Analytical Methods*

The validity of the partition assay for epoxide hydrolase was assessed by comparing hydration rates obtained here with those measured by the general tlc method of Jerina *et al.* (17). Incubations as above were terminated by vortexing with 25 μl of tetrahydrofuran and then placing on ice. Aliquots (30 μl) of each sample were applied to the loading zone of Whatman LK5DF silica gel plates, developed in toluene–propanol (10:1) and the zones cochromatographing with authentic diol and epoxide standards scraped and analyzed by lsc. The glutathione *S*-epoxide transferase (EC 2.5.1.18) contribution to the overall turnover of the candidate radiolabeled epoxide in the hepatic cytosolic fraction was measured concurrently with epoxide hydrolase simply by incorporating 5 mM glutathione (Sigma, prepared daily) in the incubation mix and analyzing the loading zone resulting from the tlc method by lsc.

### *Enzyme Preparation*

Washed microsomes (100,000$g$ pellet) and cytosolic (100,000$g$ soluble) fractions were prepared from the hepatic tissues of male Swiss–Webster mice, Sprague–Dawley rats, and a Hartley guinea pig in sodium phosphate buffer (pH 7.4, $I = 0.2$) as described (14). Protein was assayed by the method of Lowry *et al.* (29) with bovine serum albumin (Calbiochem, fraction V) as standard.

## RESULTS

A method had been sought for radiolabeling a *trans*-β-alkylstyrene oxide since they are preferred over the *cis*-isomer as substrates for the cytosolic epoxide hydrolase (L. Hasegawa *et al.*, unpublished data). Preliminary work had established the scheme of Marshall and Prager (30) as a practical route for radiolabeling β-methylstyrene oxide. This scheme involves the elemental bromination of 1-phenyl-2-propanone to form the corresponding 1-bromoketone followed by sodium borohydride reduction and subsequent ring closure with base to give a mixture of *trans*- and *cis*-β-methylstyrene oxides ($t/c = 40/60$ by NMR, glc in this study). A similar route starting with 1-phenyl-1-propanone is unsatisfactory because it is stereoselective for the less desired *cis*-isomer ($t/c = 8/92$). However, the β-methylstyrene oxides are particularly difficult to separate by hplc, and both are more volatile and less favorable than *trans*-β-ethylstyrene oxide as substrates for the mouse liver enzyme. Thus, a similar approach (30) was attempted on 1-phenyl-2-butanone. In contrast to the high yield obtained when 1-phenyl-2-propanone was used, elemental bromination of this compound proceeded with too many side reactions. However, free radical bromination with *N*-bromosuccinimide in the presence of t-butyl hydroperoxide gave high yields of the desired bromoketone.

The sequential reduction of the bromoketone (**B**) with trace sodium borohydride, borotritide, and then borohydride assured high incorporation (>95%) of tritium in the resulting epoxide mixture. A high overall

radiochemical yield was expected since a pilot synthesis with sodium borodeuteride had demonstrated >98% (by NMR) incorporation of deuterium at carbon 2.

The octanol–water partition coefficients ($P$) for *trans*-$\beta$-ethyl styrene oxide and its glycol are sufficiently different (log $P$: epoxide–3.46; diol–1.20) to allow facile partitioning of the two by the appropriate solvent system. A subsequent screen of various organic solvents as candidate hyperphases in a biphasic system (Table 1) clearly demonstrates that isooctane efficiently extracts (at a 99.9% level) the substrate epoxide from an aqueous mix while retaining 92% of the diol in the aqueous phase. Thus, very low background levels of radioactivity due to the substrate are achieved with this simple partition system allowing low levels of conversion (e.g., <1% of substrate) to be measured. A tertiary system including methanol did not significantly improve the differential partitioning of substrate and product diol as was the case in a previous assay (24).

The partition characteristics of the diol and epoxide were not effected (i.e., <2% change in isooctane/buffer distribution of diol; <0.2% for epoxide) by the following alterations of the aqueous phase: increasing the ethanol concentration to 14%, v/v, increasing the ionic strength to 0.7, phosphate instead of Tris buffer ($I$ = 0.2), and increasing bovine serum albumin concentration up to 10 mg/ml. Also, the partitioning of epoxide was not effected by aqueous levels of ethylene glycol (25%) and the nonionic detergent BRIJ 35 (1%, w/v), or by boiled microsomes at levels up to 2% tissue equivalents in the incubation. Nevertheless, while epoxide partitioning was not altered at $I$ = 1.2 (Tris + KCl), there was a 3.9% increase of diol distributing into the organic phase.

With a suitable partition system defined for quantifying both the $\beta$-ethylstyrene oxide and glycol present in an aqueous mixture, steps were undertaken to adapt the procedure into an enzymatic assay. Mouse liver cytosol was the enzyme source of choice because of the high content of epoxide hydrolase known to be present in this preparation (15). A substrate concentration ($5 \times 10^{-4}$ M) later shown to be sufficient to saturate the enzyme (Fig. 2) was used in all preliminary experiments. *Vigorous* vortexing of the enzyme with isooctane efficiently terminated the enzymatic reaction, with no additional formation of diol detected when the incubation mix thus treated was allowed to stand at room temperature for up to 2 h. Nevertheless, terminated incubations were routinely placed on ice until aliquots of each phase were removed for quantification by lsc.

Substrate volatility at 37°C (routine incubation temperature) was also investigated. Loss of both the *cis*- and *trans*-$\beta$-ethyl-

TABLE 1

PARTITIONING OF *trans*-$\beta$-ETHYLSTYRENE OXIDE AND ITS DIOL BETWEEN TRIS BUFFER AND VARIOUS ORGANIC SOLVENTS[a]

| Solvent | Percentage epoxide in organic phase[b] | Percentage diol in aqueous phase[b] |
|---|---|---|
| Isooctane | 99.87 | 92.0 |
| Isooctane–40% methanol[c] | 99.75 | 94.0 |
| Cyclohexene | 99.85 | 56.0 |
| Xylene | 99.92 | 39.0 |
| Toluene | 99.91 | 34.6 |
| Benzene | 99.97 | 29.3 |
| *n*-Octanol | 99.94 | 6.1 |

[a] Radiolabeled *trans*-$\beta$-ethylstyrene oxide or diol were introduced individually with the appropriate carrier in 1 μl ethanol to 50 μl of Tris–HCl (I = 0.2, pH 7.4) buffer such that the final concentration was $5 \times 10^{-4}$ M for the epoxide and $5 \times 10^{-5}$ M for the diol. The candidate solvent (100 μl) was added, the mixture vortexed at room temperature, and the resulting phases monitored by lsc as described under Methods.

[b] Data presented as the $\bar{x}$ for two to nine determinations.

[c] Up to 40% methanol does aid mixture of isooctane and water, and could be used to stop the enzymatic reaction if desired.

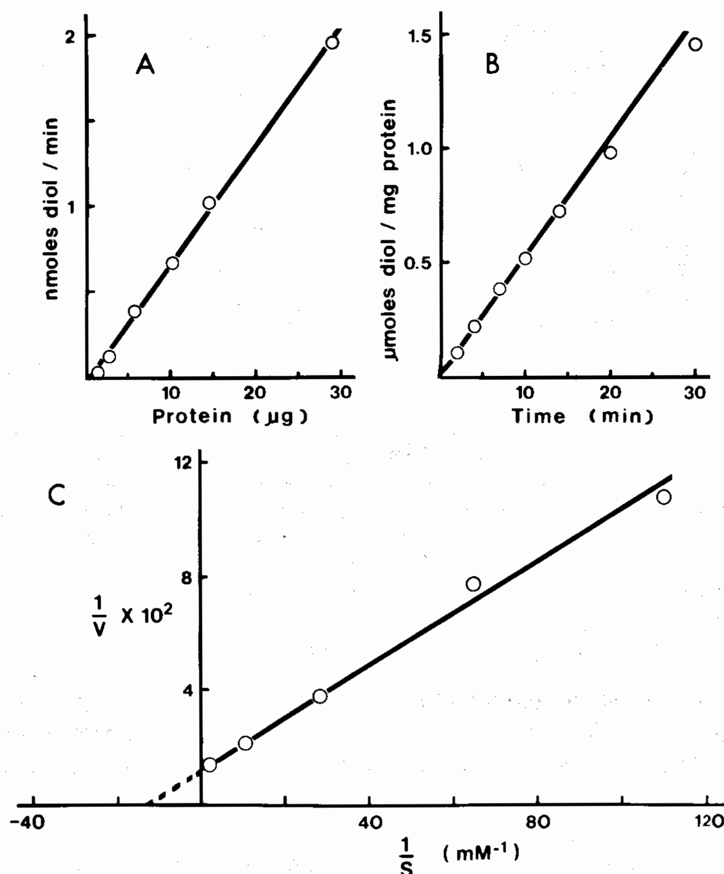


FIG. 2. Kinetics of the hydration of *trans*-β-ethylstyrene oxide by mouse cytosolic epoxide hydrolase. Epoxide hydrolase activity was measured at 37°C by the partition assay and always corrected for boiled enzyme controls. (A) Protein–velocity (10-min incubation) and (B) time–velocity (5.8 μg protein/incubation) data were both from triplicate incubations at $5 \times 10^{-4}$ M substrate. (C) Lineweaver–Burk plot where each initial velocity ($V$) in nanomoles diol formed/min-mg protein (18 incubations, time variable) was a mean of determinations made for two different protein concentrations (1.45 and 4.06 μg/incubation) each at five substrate concentrations ($S$) from $9.2 \times 10^{-6}$ to $5 \times 10^{-4}$ M. From this plot a $K_m$ of $7.2 \times 10^{-5}$ M and a $V_{max}$ of 65.1 nmol/min-mg protein were obtained.

styrene oxides from the reaction mix was only significant when incubations were extended beyond 10 min with the *cis*-epoxide being lost faster than the *trans* ($t_{1/2} \sim 29$ min, 50 min, respectively). Thus, saturating substrate was always maintained throughout routine incubations of 10 min.

With the conditions now established for monitoring diol resulting from the enzymatic cleavage of β-ethylstyrene oxide, studies were performed to test the applicability of the highly pure *cis*- and *trans*-isomers of this epoxide as substrates for epoxide hydrolase in biological systems. A screen of hepatic levels of cytosolic and microsomal β-ethylstyrene oxide hydrolase in various mammals (Table 2) confirmed the results of previous studies in this laboratory ((14), L. Hasegawa *et al.*, unpublished data) that mouse liver has an extremely active cytosolic epoxide hydrolase for *trans*-epoxides. Both geometric isomers of β-ethylstyrene oxide were metabolized faster in the cytosol than in washed microsomes,

TABLE 2

SCREEN OF HEPATIC LEVELS OF CYTOSOLIC AND MICROSOMAL β-ETHYLSTYRENE OXIDE HYDROLASE IN THREE MAMMALIAN SPECIES USING THE PARTITION ASSAY[a]

| Subcellular fraction and species | Protein[b] | *cis*-Epoxide | | *trans*-Epoxide | |
|---|---|---|---|---|---|
| | | pH 7.4 | pH 9.0 | pH 7.4 | pH 9.0 |
| Washed microsomes | | | | | |
| Rat | 20 | nd[c] | 0.4 | nd | 0.0 |
| Guinea pig | 16 | 29 | 50 | 70 | 130 |
| Mouse | 10 | 11 | 8 | 38 | 11 |
| Cytosol | | | | | |
| Rat | 49 | 0.6 | nd | 17 | nd |
| Guinea pig | 56 | 39 | 180 | 605 | 258 |
| Mouse | 56 | 150 | 75 | 3950 | 1100 |

[a] Epoxide hydrolase activities were measured by the partition assay as described under Methods and are expressed as picomoles diol formed/min-mg tissue equivalent relative to boiled enzyme controls. Each value is a mean of three to six incubations.

[b] Protein (μg/mg tissue equivalent) is expressed relative to the wet weight of the original liver homogenate.

[c] Not determined.

with hepatic activity in mouse much higher than in the guinea pig while the conversion in rat liver of this epoxide was barely above background. In addition, *cis*-β-ethylstyrene oxide is a much poorer substrate than the *trans*-isomer for the cytosolic enzyme, a trend previously confirmed in this laboratory for the β-methylstyrene oxide isomers (L. Hasegawa *et al.*, unpublished data).

Various kinetic parameters were established for the hydration of *trans*-β-ethylstyrene oxide by mouse liver cytosolic epoxide hydrolase (Fig. 2). Hydration was linear with crude cytosolic protein and time up to 30 μg protein and 14 min, respectively. Normal saturating enzyme kinetics (31) is observed for the crude soluble enzyme with an apparent $V_{max}$ and $K_m$ of 65.1 nmol/min-mg protein and $7.2 \times 10^{-5}$ M, respectively, being measured. Clearly, *trans*-β-ethylstyrene oxide is an excellent substrate for cytosolic epoxide hydrolase since both efficacy of hydration and reasonably high affinity for the enzyme is observed. An average specific activity of $69.1 \pm 7.1$ nmol/min-mg protein (SD, $n = 4$) is among the highest rates measured for any epoxide in either the cytosol or microsomes of mammalian liver (13,17,21,32). The pH optimum for *trans*-β-ethylstyrene oxide hydrolase is broad, occurring below pH 7.7 in both Tris and phosphate buffers for this (data not shown) and other substrates of the cytosolic enzyme (14,15). Nonenzymatic hydrolysis (37°C, 10 min) of *trans*-β-ethylstyrene oxide was not observed (<0.2% of epoxide present) with the partition assay in the pH range from 7–9 and amounted to only 0.8 and 5.6% of the total substrate present at pH 6.6 and 5.8, respectively.

The utility of the partition assay will obviously be restricted if pathways other than epoxide hydration are significant, and a major reaction to be expected in hepatic cytosol of mammals is GSH transferase activity (33–35). Studies were thus performed to evaluate the role of both nonenzymatic and enzymatic GSH conjugation. Both activities were monitored concurrently by tlc, and from the results shown in Table 3, the following conclusions are drawn. First, the absence or presence of glutathione had no effect on the rate of

TABLE 3

MOUSE LIVER CYTOSOLIC EPOXIDE HYDROLASE AND GLUTATHIONE *S*-TRANSFERASE ACTIVITIES FOR $\beta$-ETHYLSTYRENE OXIDE IN THE ABSENCE AND PRESENCE OF EXOGENOUS GLUTATHIONE[a]

| Incubation mix | Glutathione transferase | | Epoxide hydrolase | |
|---|---|---|---|---|
| | *cis* | *trans* | *cis* | *trans* |
| Buffer + GSH | 0.00 | 0.55 | 0.14 | 0.27 |
| Boiled cytosol − GSH | 0.00 | 0.00 | 0.10 | 0.00 |
| Boiled cytosol + GSH | 0.00 | 0.58 | 0.10 | 0.13 |
| Undialyzed cytosol − GSH | nd[b] | 0.32 | nd | 23.1 |
| Undialyzed cytosol + GSH | 0.73 | 20.7 | 0.30 | 23.9 |
| Dialyzed[c] cytosol − GSH | 0.00 | 0.28 | 0.55 | 23.1 |
| Dialyzed cytosol + GSH | 0.75 | 18.8 | 0.43 | 24.3 |

[a] Freshly prepared mouse liver cytosol was dialyzed overnight against two changes of isolation buffer and then assayed concurrently with an undialyzed control for GSH transferase and epoxide hydrolase activities in the presence or absence of 5 mM glutathione using the tlc assay as described under Methods. Data are means for at least triplicate incubations of 5 min at 37°C in pH 7.4 Tris–HCl buffer except for buffer controls where 10-min incubations were used. Activities are expressed as nanomoles product formed/min-ml incubation mix all at one dilution of enzyme under saturating substrate conditions and were corrected for nonenzymatic contributions.

[b] Not determined.

[c] Corrected for any volume change following dialysis.

enzymatic epoxide hydration in mouse liver cytosol indicating that two separate enzymes are involved for the transferase and epoxide hydrolase activities, and only in the presence of exogenous glutathione is there sufficient production of polar metabolites which would interfere with the partition assay. Second, the production of polar metabolites as the possible outcome of the presence of endogenous glutathione in the mouse liver cytosol ranged from 0.3–1.5% of the epoxide hydrolase activity, and this low interference with the partition assay was not improved by prior dialysis of the cytosol. Third, *trans*-$\beta$-ethylstyrene oxide is much better than the *cis*-substrate for both the transferase and hydrolase in mouse liver cytosol at either pH 7.4 (Table 3) or 9.0 (data not shown). Fourth, nonenzymatic GSH conjugation of $\beta$-ethylstyrene oxide is minimal at pH 7.4, but there is a decrease in enzymatic conjugation and nonenzymatic conjugation increases to almost 30% of the total at pH 9.0.

Evidence that the GSH-dependent polar metabolite(s) is, in fact, a GSH conjugate includes the following: all radioactivity of the putative conjugate cochromatographs on silica gel with a chemically synthesized epoxide conjugate (ninhydrin positive, uv absorbing at 254 nm) in solvent systems (e.g., *n*-propanol–2 N $NH_4OH$, 3:1) known to move epoxide conjugates off the origin (c.f. (33,34)). No other metabolites of $\beta$-ethylstyrene oxide were observed in this study.

## DISCUSSION

The procedure used for radiolabeling *trans*-$\beta$-ethylstyrene oxide is clearly applicable to a wide range of potential substrates for both cytosolic and microsomal epoxide hydrolases. For instance, desyl chloride (2-chloro-2-phenylacetophenone) was readily reduced and cyclized by similar methods to give a *trans*-rich mixture of stilbene oxides which are potentially useful substrates (16). The material synthesized for this study could be used in highly sensitive assays by reducing the amount of unlabeled substrate added. Since carrier-free sodium borotritide is commercially available, a similar pathway could yield a substrate useful in assays several orders of magnitude more sensitive.

One criterion for a reliable partition assay is that it generates data equivalent with other independent methods of analysis. Measurements of the hydration rates for *trans*-$\beta$-ethylstyrene oxide by mouse liver cytosol made with either the partition or tlc assay were not significantly different (63.1 ± 3.6 and 56.8 ± 4.0 nmol/min-mg protein, respectively; $n = 8$) and, indeed, a glc assay for the same substrate gives equivalent (±10%) results (K. Ota, L. Hasegawa, unpublished data). A second criterion is that competing routes of metabolism do not interfere with the partition assay. Mouse

liver cytosol is, in this respect, an excellent mammalian tissue source for developing a partition assay in that it harbors high levels of both epoxide hydrolase (12) and glutathione transferase (35,36), the latter being the primary interfering metabolic route for the partition assay.

Although *trans*-$\beta$-ethylstyrene oxide is also a good substrate for the GSH transferase, the present work reinforces the view based on former work in this laboratory (12) that the cytosolic epoxide hydrolase activity observed in mammalian liver is not due to artifacts involving GSH. The presence of GSH does not effect the rate of enzymatic epoxide hydration in mouse liver cytosol, and only in the presence of exogenous GSH does a significant interference due to transferase activity occur with this partition assay.

The major drawback of the partition assay is that it is another endpoint assay for epoxide hydrolase. However, continuous assays for epoxide hydrolase are few (37,38), can suffer from various interferences such as high nonenzymatic conversion (39), and the assays currently available do not appear suitable for studying the cytosolic enzyme. This assay is an extremely rapid and sensitive means for measuring cytosolic epoxide hydrolase in the presence of microsomal epoxide hydrolase, particularly in the mouse. *trans*-$\beta$-Ethylstyrene oxide is a poor substrate for microsomal epoxide hydrolase, a fact predictable from former substrate specificity studies (40), but surprisingly, *cis*-$\beta$-ethylstyrene oxide is also a poor substrate for the microsomal enzyme. However, the hydration of these substrates by guinea pig microsomes indicates that appropriate controls should be run before the substrate is used with other species. Rapid assays for epoxide hydrolase and GSH transferase based on the same substrate will aid considerably in elucidating the role cytosolic enzymes play in the disposition of epoxides within organisms.

## ACKNOWLEDGMENTS

The authors wish to thank K. Ota and L. Hasegawa of this division and A. Sylwester formerly of this division for their helpful comments throughout this study. This work was supported, in part, by Grant 5-R01-ES01260-03 from the U. S. Public Health Service and the California Cancer Research Coordinating Committee. B. D. Hammock was supported by NIEHS Research Career Development Award 1 K04 ES00046-01.

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