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# Continuous Spectrophotometric Assays for Cytosolic Epoxide Hydrolase[1]

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Two convenient and sensitive continuous spectrophotometric assays for cytosolic epoxide hydrolase are described. The assays are based on the differences in the ultraviolet spectra of the epoxide substrates and their diol products. The hydrolysis of 1,2-epoxy-1-(*p*-nitrophenyl)pentane (ENP5) is accompanied by a decrease in absorbance at 302 nm, while the hydration of 1,2-epoxy-1-(2-quinolyl)pentane (EQU5) produces an increase in absorbance at 315.5 nm. The $K_m$, $V_{max}$ values for ENP5 and EQU5 with purified mouse liver cytosolic epoxide hydrolase were 1.7 μM, 11,700 nmol/min/mg and 25 μM, 8300 nmol/min/mg, respectively. Both substrates are hydrolyzed significantly faster than *trans*-stilbene oxide, which is currently the most commonly used substrate for measuring cytosolic epoxide hydrolase activity. No spontaneous hydrolysis of the substrates is detectable under normal assay conditions. The assays are applicable to whole tissue homogenates as well as purified enzyme preparations. *p*-Nitrostyrene oxide and *p*-nitrophenyl glycidyl ether were also examined and found to be very poor substrates for cytosolic epoxide hydrolase from mouse liver. © 1988 Academic Press, Inc.

KEY WORDS: spectrophotometric assay; epoxide hydrolase.

Humans are exposed to a multitude of potentially mutagenic, carcinogenic, and cytotoxic epoxides present in natural products and industrial chemicals, as well as arising *in vivo* as biosynthetic intermediates and in the metabolism of olefinic and aromatic xenobiotics (1). Epoxide hydrolases (EC 3.3.2.3) catalyze the addition of water to epoxides to generate 1,2-diols (2,3). Several distinct forms of epoxide hydrolase have been identified in mammalian tissues, which differ in physical properties and substrate preferences. They include a "microsomal epoxide hydrolase" that hydrolyzes a wide range of epoxidized xenobiotics including arene oxides (2,4), a "cholesterol epoxide hydrolase" (also microsomal) that hydrates cholesterol oxides (5,6), a "leukotriene $A_4$ hydrolase" (7), and a "cytosolic epoxide hydrolase" that catalyzes the hydrolysis of a variety of epoxides not on cyclic systems (3,8). Work is currently underway in a number of laboratories to better characterize the protein chemistry of these enzymes. With the cytosolic epoxide hydrolase, a major hindrance to rapid progress has been the lack of a convenient, sensitive, continuous assay for performing kinetic analyses of the enzyme, particularly in the presence of aromatic inhibitors which absorb light in the uv region. We report here the development of two new spectrophotometric substrates, *trans*-1,2-epoxy-1-(*p*-nitrophenyl)pentane and *trans*-1,2-epoxy-1-(2-quinolyl)pentane (Fig. 1), whose hydrolysis by cytosolic epoxide hydrolase may be conveniently monitored above 300 nm. They are both excellent substrates for the cytosolic enzyme and show no detectable spontaneous hydrolysis under normal assay conditions. A comparison of the new assays with the previously developed *trans*-stilbene oxide continuous spectropho-

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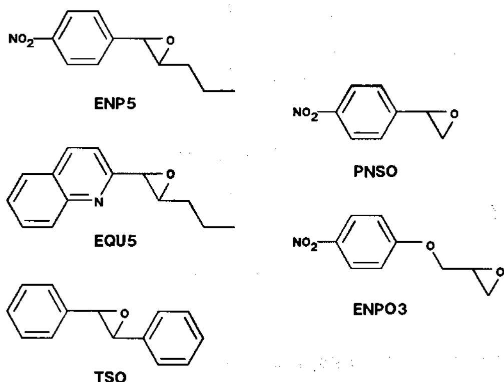


FIG. 1. Structures of spectrophotometric substrates for epoxide hydrolase.

tometric assay (9) for cytosolic epoxide hydrolase is presented. *p*-Nitrostyrene oxide (10) and *p*-nitrophenyl glycidyl ether (11), two spectrophotometric substrates previously used to measure microsomal epoxide hydrolase and glutathione *S*-transferase activity, were also evaluated for their potential as substrates for the cytosolic enzyme.

## MATERIALS AND METHODS

*Synthesis of trans-1,2-epoxy-1-(2-quinolyl)-pentane (EQU5).*[4] The procedure of Still and Novack (12) for the direct epoxidation of carbonyl compounds was applied to the synthesis of 1,2-epoxy-1-(2-quinolyl)pentane. This method provides a *trans* stereoselective synthesis of epoxides via reaction of an unstabilized arsonium ylide with an aldehyde. Quinoline 2-aldehyde (2-quinolinecarboxaldehyde) was available commercially (Chem Service, West Chester, PA) and the *n*-butyltriphenylarsonium fluoroborate was prepared in three steps from *n*-butanol.

*n*-Butyl triflate was synthesized following the general procedure of Beard *et al.* (13) for preparation of alkyl triflates. To a solution of 20.0 g (71 mmol) of trifluoromethanesulfonic anhydride (Aldrich Chemical Co., Milwaukee, WI) in 60 ml of methylene chloride at 0°C was added dropwise with stirring a solution of 4.60 g (62 mmol) of *n*-butanol and 4.90 g (0.062 mmol) of pyridine in 25 ml methylene chloride. After 20 min, the solution was washed with water, dried over sodium sulfate, and distilled under vacuum (77°C, ca. 80 mm Hg) to give 10.36 g (81% yield) of the product as a colorless oil.

For the preparation of *n*-butyltriphenylarsonium triflate (12), a solution of 10.3 g (50 mmol) *n*-butyl triflate and 18.4 g (60 mmol) triphenylarsine in 50 ml of methylene chloride was stirred at room temperature for 5 days. The solvent was removed by rotary evaporation to give a viscous oil which formed a white solid upon mashing with ether. The solid was filtered, washed with ether, and dried under vacuum, to give 23.6 g of the desired salt (92% yield).

Fluoroborate exchange was accomplished by addition in a single portion of a solution of 10.2 g (20 mmol) *n*-butyltriphenylarsonium triflate in 110 ml methanol to a vigorously stirred solution of 220 g (2 mol) of sodium tetrafluoroborate in 800 ml of water at room temperature (12). After stirring for 3 h, the reaction mixture was extracted four times with 400 ml of chloroform. The extracts were combined and dried over sodium sulfate, and the solvent was removed by rotary evaporation to give a light yellow oil, which produced 8.67 g (96% yield) of a white solid upon mashing with ether. This salt was used without further purification.

For the synthesis of *trans*-1,2-epoxy-1-(2-quinolyl)pentane, 8.57 ml (12 mmol) of potassium hexamethyldisilazide (1.4 M solution in THF, Alfa Products, Danvers, MA) was added dropwise to a solution of 6.30 g (14 mmol) *n*-butyltriphenylarsonium fluoroborate and 2 ml dry hexamethylphosphoramide in 10 ml dry THF under nitrogen at −40°C. After it was stirred at −40°C for 15 min, the solution was cooled to −78°C, and 10 ml (10 mmol) of 2-quinolinecarboxalde-

[4] Abbreviations used: EQU5, *trans*-1,2-epoxy-1-(2-quinolyl)pentane; THF, tetrahydrofuran; ENP5, *trans*-1,2-epoxy-1-(*p*-nitrophenyl)pentane; PNSO, *p*-nitrostyrene oxide; TSO, *trans*-stilbene oxide; ENPO3, 1,2-epoxy-3-(*p*-nitrophenoxy)propane.

hyde (1.0 M solution in THF) was added dropwise. The reaction mixture was stirred at −78°C for 5 min or until the aldehyde had disappeared, after which the solution was allowed to warm to room temperature and stirred for 1 h. The solvent was removed by rotary evaporation and the crude mixture was purified by flash chromatography using hexanes/ethyl acetate (9/1) to give 1.15 g (54% yield) of a golden oil. The relatively low yields generally obtained from aromatic aldehydes with the arsonium ylide procedure were previously reported by Still and Novack (12).

*Synthesis of trans-1,2-epoxy-1-(p-nitrophenyl)pentane (ENP5).* *trans*-1,2-Epoxy-1-(*p*-nitrophenyl)pentane was synthesized from *n*-butyltriphenylarsonium tetrafluoroborate and 4-nitrobenzaldehyde in the manner described above for 1,2-epoxy-1-(2-quinolyl)-pentane. The product was purified first by flash chromatography (hexanes/ethyl acetate 9/1) and then by semipreparative normal-phase HPLC (hexanes/ether 97/3) to remove contaminating 1-(*p*-nitrophenyl)-2-pentanone that cochromatographed with the epoxide on TLC and constituted approximately 30% of the originally isolated product. The identity of the 1-(*p*-nitrophenyl)-2-pentanone by-product was confirmed by $^1$H NMR ($\delta$1.0,t,3H,$CH_3$;$\delta$1.2–1.8,m,4H,–$CH_2$–$CH_2$–; $\delta$3.0,t,2H,Ar–$CH_2$–;$\delta$8.1,q,4H,arom), IR (1695 $cm^{-1}$, C=O), and mass spectrometry ($M^+$ = 207).

*Sources of other epoxide substrates and diols.* *p*-Nitrostyrene oxide (PNSO) was synthesized as described by Westkaemper and Hanzlik (14). 1,2-Epoxy-3-(*p*-nitrophenoxy)-propane (ENPO3, *p*-nitrophenyl glycidyl ether) was obtained from Eastman Kodak (Rochester, NY) and recrystallized from ethanol prior to use. *trans-p*-Nitrostilbene was synthesized as described by Wheeler and Batlle de Pabon (15) and then oxidized with *m*-chloroperoxybenzoic acid (16) to give *trans-p*-nitrostilbene oxide. *trans*-Stilbene oxide (TSO) and its *meso*-diol were purchased from Aldrich Chemical Company and MCB-Schucherdt (Gibbstown, NJ), respectively. The remaining diols were synthesized from the corresponding epoxides by hydrolysis with aqueous $HClO_4$ (3–20%) in *t*-butyl alcohol (14) and were purified by TLC (hexanes/ethyl acetate).

*Analytical procedures.* The analytical data for the epoxides and diols examined in this study are listed in Table 1. Mass spectra for the compounds were obtained on a VG ZAB-2F with a VG 11-250 data system and were consistent with their structures (25). Ultraviolet difference spectra were measured using a Varian/Cary 219 spectrophotometer and are summarized in Table 2.

*Enzyme preparation.* Cytosolic fractions were prepared from the livers of clofibrate-treated male Swiss-Webster mice as described previously (17). Purified cytosolic epoxide hydrolase was obtained by a benzylthio-Sepharose affinity chromatography procedure (18).

*Assays.* Photometric assays were conducted in a Varian/Cary 219 spectrophotometer at 25°C. Typically, 50 $\mu$l of ice-cold enzyme solution was added to sample and reference cuvettes containing 940 $\mu$l of pH 7.4 sodium/potassium phosphate buffer ($I$ = 0.2) containing 100 $\mu$g/ml bovine serum albumin (buffer A). After equilibration at 25°C, 10 $\mu$l of ethanol was added to the reference cuvette and the reaction initiated by the addition of the substrate in 10 $\mu$l of ethanol to the sample cuvette. Typical final substrate concentrations were 0.10 mM for EQU5 and 0.050 mM for ENP5. For optimal activity the EQU5 and ENP5 assays should be performed at pH 7.0 rather than pH 7.4 (which was used in this study to facilitate comparison with earlier studies). The change in absorbance was monitored at the wavelength corresponding to the $\lambda_{max}$ for the difference spectrum of the epoxide and diol (Table 2).

## RESULTS AND DISCUSSION

*Rationale for design of spectrophotometric substrates.* The development of the new ENP5 and EQU5 substrates was based on previous observations of the substrate prefer-

TABLE 1

SPECTROPHOTOMETRIC SUBSTRATE AND PRODUCT ANALYTICAL DATA

| Substrate/product | mp[a] | $R_f$[b] | | Elemental analysis[c] Calcd | Elemental analysis[c] Found | $^1$H NMR[d] | IR ($cm^{-1}$)[e] |
|---|---|---|---|---|---|---|---|
| ENP5 ($C_{11}H_{13}NO_3$)<br>*trans*-1,2-Epoxy-1-<br>(4-nitrophenyl)pentane | liq | 0.70 | C:<br>H:<br>N: | 63.76<br>6.32<br>6.76 | 63.86<br>6.42<br>6.84 | δ1.0(t, 3H, –$CH_3$), δ1.6(m, 4H, –$CH_2$–)<br>δ2.9(m, 1H, –CH–), δ3.6(d, 1H, –CH–Ar)<br>δ7.7(q, 4H, arom)<br>($CDCl_3$) | 2946, 2936, 1606,<br>1524, 1349,<br>849, 823, 750,<br>695 (neat) |
| DNP5 ($C_{11}H_{15}NO_4$)<br>1,2-Dihydroxy-1-<br>(4-nitrophenyl)pentane | 108 | 0.27 | C:<br>H:<br>N: | 58.66<br>6.71<br>6.22 | 58.66<br>6.79<br>6.09 | δ0.9(d, 3H, –$CH_3$), δ1.4(m, 4H, –$CH_2$–$CH_2$–)<br>δ2.0(d, 1H, Ar–C–C–OH), δ2.6(d, 1H, Ar–C–OH)<br>δ3.9(m, 1H, Ar–C–CH–), δ4.9(dd, 1H, Ar–CH–)<br>δ7.9(q, 4H, arom)<br>($CDCl_3$) | 3400, 3286, 2960,<br>1524, 1516,<br>1345, 1048,<br>804, 719 (KBr) |
| EQU5 ($C_{14}H_{15}NO$)<br>*trans*-1,2-Epoxy-1-<br>(2-quinolyl)pentane | liq | 0.65 | C:<br>H:<br>N: | 78.84<br>7.09<br>6.57 | 78.70<br>7.16<br>6.49 | δ1.0(t, 3H, –$CH_3$), δ1.6(m, 4H, –$CH_2$–)<br>δ3.1(m, 1H, –CH–), δ3.9(d, 1H, –CH–Ar)<br>δ7.1–8.1(m, 10H, arom)<br>($CDCl_3$) | 2961, 2933, 1601,<br>1506, 1428,<br>896, 830, 756,<br>617 (neat) |
| DQU5 ($C_{14}H_{17}NO_2$)<br>1,2-Dihydroxy-1-<br>(2-quinolyl)pentane | 106–107 | 0.25 | C:<br>H:<br>N: | 72.70<br>7.41<br>6.06 | 72.82<br>7.35<br>5.99 | δ0.9(d, 3H, –$CH_3$), δ1.4(m, 4H, –$CH_2$–$CH_2$–)<br>δ2.95(br s, 1H, –OH), δ4.0(q, 1H, Ar–C–C–H)<br>δ4.75 (br s, 1H, –OH), δ4.9(d, 1H, Ar–C–H)<br>δ7.1–8.1(m, 10H, arom)<br>($CDCl_3$) | 3341, 2950, 2920,<br>2870, 1604,<br>1088, 1048,<br>1030, 835, 753<br>(KBr) |
| TSO ($C_{14}H_{12}O$)<br>*trans*-Stilbene oxide | 65–67 | 0.71 | | — | | — | — |
| TSD ($C_{14}H_{14}O_2$)<br>*meso*-Stilbene diol | 130–131 | 0.44 | | — | | — | — |
| PNSO ($C_8H_7NO_3$)<br>*p*-Nitrostyrene oxide | 83.5–<br>85 | 0.56 | C:<br>H:<br>N: | 58.18<br>4.27<br>8.48 | 58.09<br>4.23<br>8.34 | δ2.7(dd, 1H, Ar–C–C–H), δ3.2(dd, 1H, Ar–C–C–H)<br>δ3.9(dd, 1H, Ar–C–H), δ7.8(q, 4H, arom)<br>($CDCl_3$) | 1608, 1521, 1346,<br>1316, 876, 859,<br>848, 760, 749,<br>695 (KBr) |

TABLE 1—*Continued*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| PNSD ($C_8H_9NO_4$) | 77–78 | 0.10 | C: | 52.46 | 52.08 | δ3.4(m, 3H, –CH–CH$_2$), δ4.7(m, 1H, Ar–C–C–OH) | 3398, 1536, 1352, |
| *p*-Nitrostyrene diol | | | H: | 4.95 | 4.88 | δ5.4(d, 1H, Ar–C–OH), δ7.7(q, 4H, arom) | 1095, 1069, |
| | | | N: | 7.65 | 7.53 | ($d_6$-DMSO) | 1037, 856, 825, |
| | | | | | | | 699 (KBr) |
| ENPO3 ($C_9H_9NO_4$) | 64–66 | 0.44 | | — | | — | — |
| 1,2-Epoxy-3- | | | | | | | |
| (4-nitrophenoxy)propane | | | | | | | |
| DNPO3 ($C_9H_{11}NO_5$) | liq | 0.06 | C: | 52.46 | 52.08 | δ1.7–2.7(2 br s, 2H, –OH), δ3.8(m, 2H, –CH$_2$–) | 3388, 1609, 1594, |
| 1,2-Dihydroxy-3- | | | H: | 4.95 | 4.88 | δ4.1(br s, 3H, –CH–CH$_2$), δ7.5(q, 4H, arom) | 1511, 1346, |
| (4-nitrophenoxy)propane | | | N: | 7.65 | 7.53 | ($CDCl_3$) | 1266, 1133, |
| | | | | | | | 846, 752 (neat) |

[a] Melting points were determined with a Thomas–Hoover apparatus and are uncorrected.
[b] $R_f$ values are reported for a hexane/ethyl acetate 1:1 solvent system.
[c] Elemental analyses were performed by the University of California, Berkeley, Analytical Lab.
[d] $^1$H NMR spectra were determined in deuterated chloroform or DMSO solution using a Varian EM-390 spectrometer.
[e] The ir spectra were measured either neat or in KBr pellets using an IBM 32 FT-IR.

ences of epoxide hydrolases. Styrene oxide is a good substrate for the microsomal epoxide hydrolase and a poor to moderately good substrate for the cytosolic enzyme (2–4,19,20). *trans-β*-Alkylstyrene oxides, in contrast, such as *trans-β*-ethylstyrene oxide and *trans-β*-propylstyrene oxide, are very good substrates hydrated almost exclusively by the cytosolic enzyme (19–21). A very useful spectrophotometric assay for the microsomal epoxide hydrolase utilizing *p*-nitrostyrene oxide was developed by Westkaemper and Hanzlik (10). The above results suggested that the addition of a *trans-β*-alkyl group to *p*-nitrostyrene oxide might produce a useful spectrophotometric substrate for the cytosolic enzyme. The presence of the nitro group in the substrate was also expected to increase its solubility in water and decrease the volatility problems encountered with the *trans-β*-alkylstyrene oxides. This was the basis for the synthesis of ENP5 which has proved to be an excellent substrate for the cytosolic enzyme whose hydrolysis may be monitored by a decrease in absorbance at 302 nm. Another factor considered in the design of the new substrates was the desirability of a product appearance assay in contrast to the currently available substrate disappearance assays. A necessary requirement for such a substrate would be a significant uv spectral shift on the hydrolysis of the epoxide to a diol. It was thought that by introducing the possibility of intramolecular hydrogen bonding in the product diol such a spectral shift might be obtained. This was the basis for the synthesis of EQU5 in which the ring nitrogen and *β*-hydroxy group of the corresponding diol are in a favorable position for hydrogen bonding. The quinoline moiety was also expected to provide a good uv chromophore and impart desirable water solubility characteristics, while the *trans-β*-alkyl group would accommodate the enzyme's substrate preferences. A significant spectral shift was observed (Fig. 2) and served as the basis for a convenient produce appearance assay at 315.5 nm. Alternatively, the disappearance of EQU5 may be monitored at 320

TABLE 2

ULTRAVIOLET SPECTRAL DATA FOR EPOXIDE HYDROLASE SUBSTRATES

| Substrate | Epoxide–diol difference spectrum[a] $\lambda_{max}$ (nm) | $\epsilon$ ($M^{-1}$ $cm^{-1}$) | Product appearance assay |
|---|---|---|---|
| ENP5 | 302 | 1,520 | No |
| EQU5 | 315.5 | 1,500 | Yes |
| | 320 | 3,700 | No |
| TSO | 229 | 15,000 | No |
| PNSO | 293 | 1,350 | No |
| ENPO3 | 302 | 1,560 | No |

[a] Ultraviolet spectra were measured in pH 7.4 sodium/potassium phosphate buffer, $I = 0.2$.

nm. Two other logical candidates for spectrophotometric substrates were synthesized [*trans-p*-nitrostilbene oxide and *trans*-1,2-epoxy-1-(2-pyridyl)pentane] but are not included in this study owing to undesirable uv spectral characteristics ($\lambda_{max}$ = 271 nm) in the first case and a propensity to form micelles at concentrations below 0.05 mM with the latter.

*Assay validity, linearity, and kinetics.* The diol was shown by several methods to be the only metabolite produced from EQU5 and ENP5 by either crude cytosol or the homogeneous enzyme. The uv spectrum of the assay solution following incubation of the epoxide with enzyme matched exactly that of the authentic diol. Extraction of the assay solution with ethyl ether followed by TLC analysis resulted in a single spot with an $R_f$ identical to that of the authentic diol. Both diols also reacted quantitatively with *n*-butylboronic acid (a diol-selective reagent) to yield a single spot on TLC of higher $R_f$. The EQU5 and ENP5 spectrophotometric assays were demonstrated to be linear with time and protein concentration (EQU5: 0–12 min, 0–1 μg purified enzyme; ENP5: 0–8 min, 0–0.5 μg purified enzyme) (25). For EQU5 an apparent $K_m$ of 25 μM and a $V_{max}$ of 8300 nmol/min/mg were determined at 25°C using purified cytosolic epoxide hydrolase from livers of clofibrate-treated mice (Fig. 3A). An apparent $K_m$ of 1.7 μM and a $V_{max}$ of 11,700 nmol/min/mg were determined for ENP5 (Fig. 3B). These results compare with values of 5 μM and 3300 nmol/min/mg measured at 37°C for TSO with the purified mouse liver enzyme (20).

*Effect of pH on EQU5 hydration.* The pH optimum for EQU5 hydrolysis by purified cytosolic epoxide hydrolase from mouse liver is shown in Fig. 4A. Maximal activity was observed near pH 7, which coincides with the pH optimum determined for TSO (20,22). Molar extinction coefficients for the difference spectrum of EQU5 and its diol were determined at each pH value (Fig. 4B) and were found to increase with decreasing pH, which is attributable to the strong hyperchromic effect that results from protonation of a quinoline nitrogen (23).

*Comparison with other epoxide hydrolase spectrophotometric substrates.* The rates of enzymatic hydrolysis for the five spectrophotometric substrates examined in this study are listed in Table 3. ENP5 and EQU5 are hydrated by the cytosolic epoxide hydrolase at rates approximately 15-fold and 5-fold higher

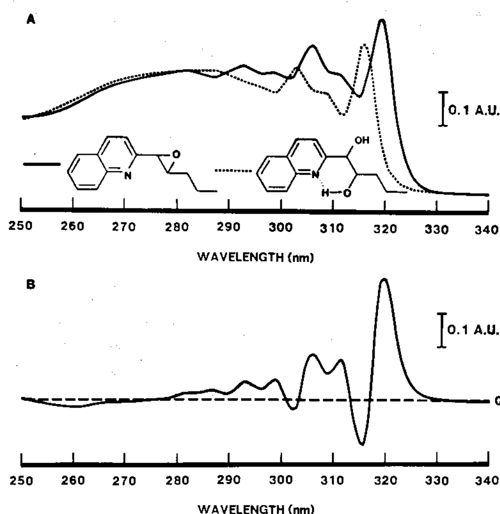


FIG. 2. Ultraviolet spectra of EQU5, its corresponding diol, and their difference spectrum. All spectra were measured at a concentration of 0.050 mM in pH 7.4 sodium/potassium phosphate buffer ($I$ = 0.2). The presence of bovine serum albumin at 100 μg/ml had no effect on the absorbance spectra.

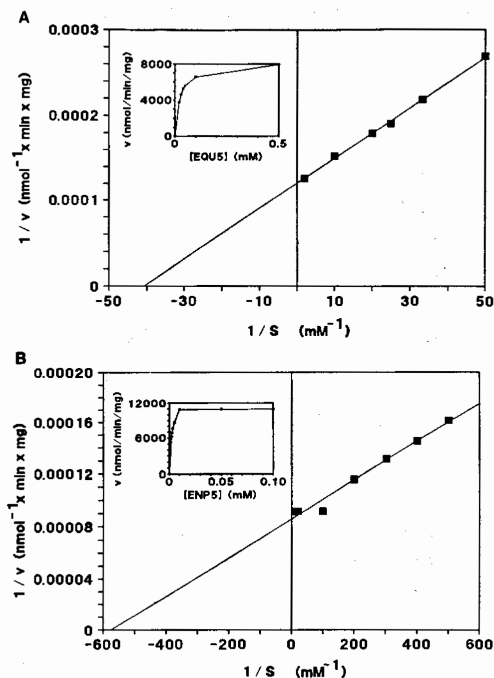


FIG. 3. Kinetic analysis of EQU5 and ENP5 hydration by purified mouse liver cytosolic epoxide hydrolase. Initial rates were determined at 25°C under standard assay conditions with 0.46 $\mu$g/ml (EQU5) and 0.046 $\mu$g/ml (ENP5) of purified enzyme. Each data point represents the mean of at least three determinations. The experiment was repeated with similar results.

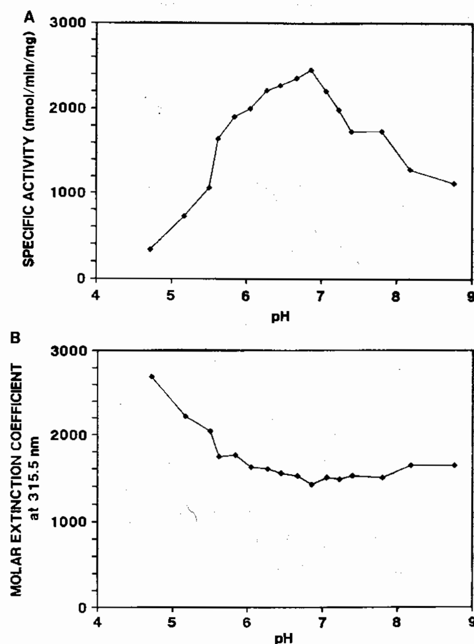


FIG. 4. Effect of pH on the hydration of EQU5. (A) Hydration of EQU5 (0.10 mM) by 0.046 $\mu$g/ml of purified mouse liver cytosolic epoxide hydrolase at 25°C. Assays were performed in $I$ = 0.2 buffers with no bovine serum albumin added: sodium acetate (pH 4.7–5.6), sodium/potassium phosphate (pH 5.6–7.4), and Tris–HCl (pH 7.4–8.8). The data points represent the means of triplicate determinations. (B) Molar extinction coefficient at 315.5 nm for the difference spectrum of EQU5 and its diol.

than that of TSO. No detectable hydrolysis of ENP5 or EQU5 was observed in crude microsomal preparations from mouse liver at protein concentrations up to 800 $\mu$g/ml, suggesting that the new substrates exhibit the same selectivity for the cytosolic enzyme as observed for the *trans*-$\beta$-alkylstyrene oxides. PNSO and ENPO3 have been suggested previously as possible spectrophotometric substrates for the cytosolic epoxide hydrolase (3,9). PNSO is currently used to monitor purified microsomal epoxide hydrolase activity spectrophotometrically at 310 nm (10), while ENPO3 is a commercially available substrate for glutathione *S*-transferase whose conjugation can be monitored by an increase in absorbance at 360 nm (11). Both PNSO and ENPO3, however, appear to be poor substrates for the cytosolic enzyme from mouse liver (Table 3). This indicates that PNSO might be very useful in conjunction with EQU5 or ENP5 for monitoring both epoxide

TABLE 3

HYDRATION OF SPECTROPHOTOMETRIC SUBSTRATES BY EPOXIDE HYDROLASE FROM MOUSE LIVER[a]

| Substrate | Cytosol | Purified cytosolic epoxide hydrolase |
|---|---|---|
| ENP5 | 129 ± 1 | 10,900 ± 50 |
| EQU5 | 45.5 ± 0.6 | 3,240 ± 10 |
| TSO | 8.86 ± 0.05 | 677 ± 13 |
| PNSO | 1.45 ± 0.10 | 16.3 ± 0.1 |
| ENPO3 | 0.13 ± 0.01 | 6.34 ± 0.02 |

[a] Initial rates of hydration (nmol/min/mg ± SEM) at 25°C and 0.050 mM substrate concentration in buffer A.

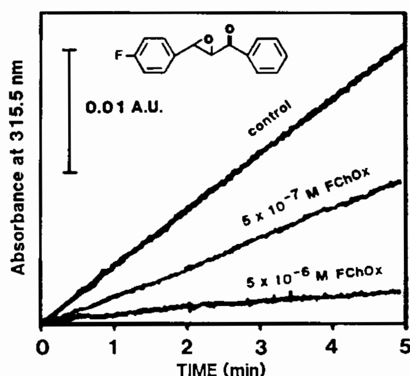


FIG. 5. Inhibition of EQU5 hydration by a chalcone oxide. The 4-fluorochalcone oxide (FChOx) inhibitor was added in 10 μl of ethanol to 980 μl of purified mouse liver cytosolic epoxide hydrolase (0.093 μg/ml) and preincubated with the enzyme for 10 min at 25°C prior to the addition of EQU5. Less than 5% variation in rates was observed on repetition of the experiment.

hydrolases important in the hydration of xenobiotics. Similarly, ENPO3 could prove useful for monitoring some of the glutathione *S*-transferase isozymes even in the presence of the cytosolic epoxide hydrolase.

*Advantages and limitations of the spectrophotometric assays for cytosolic epoxide hydrolase.* The TSO, ENP5, and EQU5 assays all offer the advantages of sensitivity and stability, with ENP5 being the most sensitive. TSO is commercially available, while ENP5 and EQU5 require a four-step synthesis for their preparation. The low aqueous solubility (0.1 mM) of TSO is a limitation that is less of a problem with the more water-soluble ENP5 and EQU5 substrates. TSO hydration is monitored at a wavelength of 229 nm, where most aromatic compounds absorb strongly, whereas the hydration of ENP5 and EQU5 are monitored above 300 nm, which allows convenient evaluation of most aromatic inhibitors and activators. The utility of EQU5 in examining the inhibition of cytosolic epoxide hydrolase by a chalcone oxide is demonstrated in Fig. 5. Such an experiment would prove very difficult with TSO due to the high extinction coefficient of the inhibitor. EQU5 was also used to examine the effect of T-2 toxin (a 12,13-epoxytrichothecene) on purified cytosolic epoxide hydrolase activity. At concentrations up to 0.5 mM, no inhibition or activation of enzyme activity was observed, as expected from the lack of hydration of radioactive T-2 toxin by the enzyme. The EQU5 spectrophotometric substrate has the additional advantage of providing a product appearance assay.

*Further applications.* The EQU5 and ENP5 spectrophotometric assays could offer dramatic time savings if adapted for use in kinetic microtiter plate readers likely to be available in the near future that are modified to operate at wavelengths down to 300 nm (24). These substrates should also be suitable for the development of assays that simultaneously monitor cytosolic epoxide hydrolase and glutathione *S*-transferase activities following separation of the products by partition-, TLC-, or HPLC-based methods.

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