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SPECTROPHOTOMETRIC ASSAY FOR MAMMALIAN CYTOSOLIC EPOXIDE HYDROLASE USING *TRANS*-STILBENE OXIDE AS THE SUBSTRATE

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Abstract—A continuous spectrophotometric assay based on the differences in the ultraviolet spectra of *trans*-stilbene oxide (TSO) and its reaction product 1,2-diphenyl-1,2-ethanediol is described for the measurement of mammalian cytosolic epoxide hydrolase activity. Rates of TSO hydration determined by this method were similar to those obtained by independent analytical methods, and the assay is rapid, reproducible and relatively free from interference.

Mammals employ a number of mechanisms to detoxify and/or rid themselves of exogenous compounds. Among these mechanisms are enzymes which catalyze the hydration of epoxides to diols (epoxide hydrolase, EC 3.3.2.3) and enzymes which catalyze the conjugation of glutathione (GSH) to compounds containing electrophilic sites (glutathione transferases, EC 2.5.1.18). Epoxide hydrolase activity is associated not only with the microsomal and nuclear membranes [1] but also with the cytosolic fraction and the mitochondrial lumen [2–5]. The subcellular fraction in which a particular enzyme activity occurs may vary, depending upon numerous factors. However, at least two distinct enzymes exist which have different physical properties and substrate preferences. For convenience they are referred to here as microsomal and cytosolic epoxide hydrolases.

Both enzymes are clearly important in the hydration of natural and foreign compounds, and rapid assays are needed for a number of pharmacological studies. A variety of assays exist for the microsomal hydrolase; however, many of these assays are not appropriate for the cytosolic enzyme (for review see Ref. 2). Much of the information on the cytosolic epoxide hydrolase has been obtained by assays based on thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) [2, 6]. Further study on the enzyme has been facilitated by the development of rapid and sensitive radiometric partition assays [7, 8]; all of these assays, however, suffer from being end-point assays. A direct and sensitive continuous assay would thus be useful for kinetic studies on the cytosolic epoxide hydrolase.

Substrate specificity studies have identified a number of substrates rapidly hydrated by the cytosolic enzyme, but only poorly hydrated by the microsomal enzyme ([9], manuscript in preparation). Among these substrates was one with interesting pharmacological properties [10–12] and favorable spectrophotometric characteristics, *trans*-stilbene oxide. This paper describes the development of a continuous spectrophotometric assay for mammalian cytosolic epoxide hydrolase, and its possible application to the study of glutathione epoxide transferase.

MATERIALS AND METHODS

Chemicals. trans-Stilbene oxide (TSO) and its pure *meso*-diol were purchased from the Aldrich Chemical Co., Milwaukee, WI, and MCB-Schucherdt, Gibbstown, NJ, respectively. 1,2-Diphenyl-1,2-ethanediol (60:40 *threo/meso* mixture) was prepared by hydration of TSO with acidic aqueous tetrahydrofuran [13] and was purified by Florisil and then silica gel column chromatography followed by crystallization from ethanol. Reduced glutathione and BSA fraction V were obtained from the Sigma Chemical Co., St. Louis, MO. [^{3}H]-*trans*-Stilbene oxide was either a gift of F. Oesch, University of Mainz, Germany [14], or it was prepared in this laboratory by the reduction of desyl chloride with sodium borohydride [^{3}H] (S. S. Gill, manuscript in preparation). None of the substrates used was optically active. Butylated hydroxyanisole (BHA) diet was provided by A. Poland, University of Wisconsin, Madison, WI.

Animals and enzymes. Male Swiss–Webster mice, 7 weeks old, and male Sprague–Dawley rats, ~150 g, were obtained from the Simonsen Laboratories, Gilroy, CA. Control animals were fed Purina rodent chow, and the induced animals were fed a similar diet containing 0.75% BHA by weight for 11 days prior to being killed. Liver cytosolic fractions (100,000 *g* supernatant fractions) were prepared in potassium phosphate buffer (pH 7.4, I = 0.2) as described previously [6].

Spectrophotometric assays. All ultraviolet (u.v.) spectra were taken on a Beckman 25 spectrophotometer at 25°. Decrease in absorbance at 229 nm due to loss of TSO was monitored with a Varian Cary 219 spectrophotometer thermostatted at 37° using the double-beam mode. All spectral measure-

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ments were made in potassium phosphate buffer (pH = 7.4, I = 0.2 M) unless otherwise noted.

Cytosolic epoxide hydrolase and glutathione transferase were assayed in a final volume of 2 ml. After equilibration of buffer in the sample and reference cuvettes to 37° (5 min), ice-cold liver cytosol [100 μl of 5% (w/v) tissue equivalents] was added to both cuvettes, the spectrophotometer was optically balanced, and 20 μl of ethanol was placed in the reference cuvette. The reaction was then initiated by the addition of 20 μl of TSO in ethanol (5×10^{-3} M) to the sample cell with thorough mixing (insufficient mixing resulted in a lag period). Unless otherwise specified, protein concentration was between 0.15 and 0.20 mg/ml as determined by the Warburg–Christian method [15]. Glutathione transferase activity was followed similarly except that 100 μl of the appropriate ice-cold, freshly prepared GSH solution was also added once the initial rate of epoxide hydrolase activity had been determined.

RESULTS

trans-Stilbene oxide (TSO) is both an excellent substrate for the cytosolic epoxide hydrolase ([2, 4, 9, 16], unpublished data) and a strongly u.v. absorbing molecule, and thus it could be a suitable substrate in a spectrophotometric assay for this enzyme. The u.v. spectrum for TSO (Fig. 1A) had two λ_{max}, 228 nm (ε 20,800 $M^{-1}cm^{-1}$) and 210 nm (ε 14,400 $M^{-1}cm^{-1}$). The spectrum of 1,2-diphenyl-1,2-ethanediol (λ_{max} 215 nm, ε 17,000 $M^{-1}cm^{-1}$) indicated that the hydration of the epoxide to the diol was accompanied by a spectral shift of sufficient magnitude to make absorption of the diol insignificant at 229 nm, the λ_{max} of the difference spectrum (Fig. 1B). The spectra of the *meso*- and *threo*-diols were superimposable. Unfortunately, the spectrum of *cis*-stilbene oxide (λ_{max} 210 nm, ε 16,700 $M^{-1}cm^{-1}$) was very similar to that of the diol, and interference due to diol at 226 nm, the λ_{max} of the difference spectrum, would be significant.

When hexane was used as the solvent, the u.v. spectra of TSO and 1,2-diphenyl-1,2-ethanediol were unchanged but, with Tris buffer at pH 7.4 (I = 0.2) as the solvent, TSO had a λ_{max} at 229 nm and lost the 210 nm peak. The spectra of *cis*-stilbene oxide and the diol both had λ_{max} at 221 nm. Tris buffer at pH 9.0 (I = 0.1) affected the spectra of these compounds to a greater extent: there was a shift of λ_{max} to longer wavelengths and a decrease in the absorbance which was slight in the case of TSO (λ_{max} 232 nm, ε 18,800 $M^{-1}cm^{-1}$).

The effect of protein concentration on the u.v. spectra of TSO and 1,2-diphenyl-1,2-ethanediol is shown in Fig. 2. As the protein concentration increased, there was a change in the spectra characterized by a shift of λ_{max} to the longer wavelengths and a decrease in absorbance that was more pronounced in the diol spectrum than in the epoxide. The double peaks of TSO became a single maximum as the protein concentration increased from 0 to 0.15 mg/ml. There was also the possibility that a contaminant responsible for the 210 nm peak was obscured by the increase in protein concentration. The 210 nm peak, however, remained following multiple crystallization of TSO from hexane [17].

Addition of exogenous BSA was used to further investigate the effect protein has on the u.v. absorbance of TSO and its diol. At 229 nm the absorbance of TSO decreased slightly as the protein concentration increased (Fig. 3A). This decrease, which was partially due to a slight shift in λ_{max}, was insignificant within the protein concentrations (0.15 to 0.33 mg/

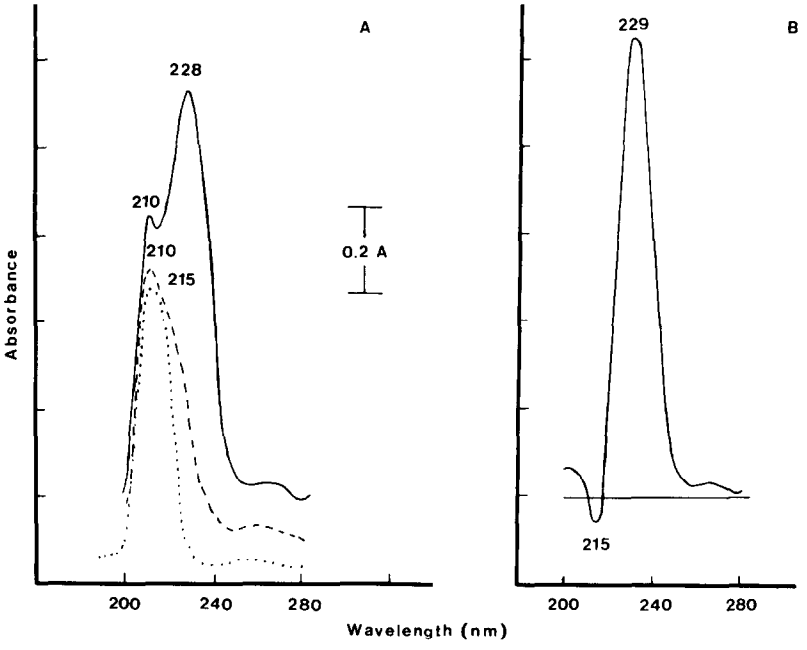

Fig. 1. Ultraviolet spectra in phosphate buffer (pH 7.4, I = 0.2) of 5×10^{-5} M (A) TSO (——), *cis*-stilbene oxide (- - -) and 1,2-diphenyl-1,2-ethanediol (· · ·), and (B) the difference spectrum of 5×10^{-5} M TSO against 5×10^{-5} M 1,2-diphenyl-1,2-ethanediol as the reference.

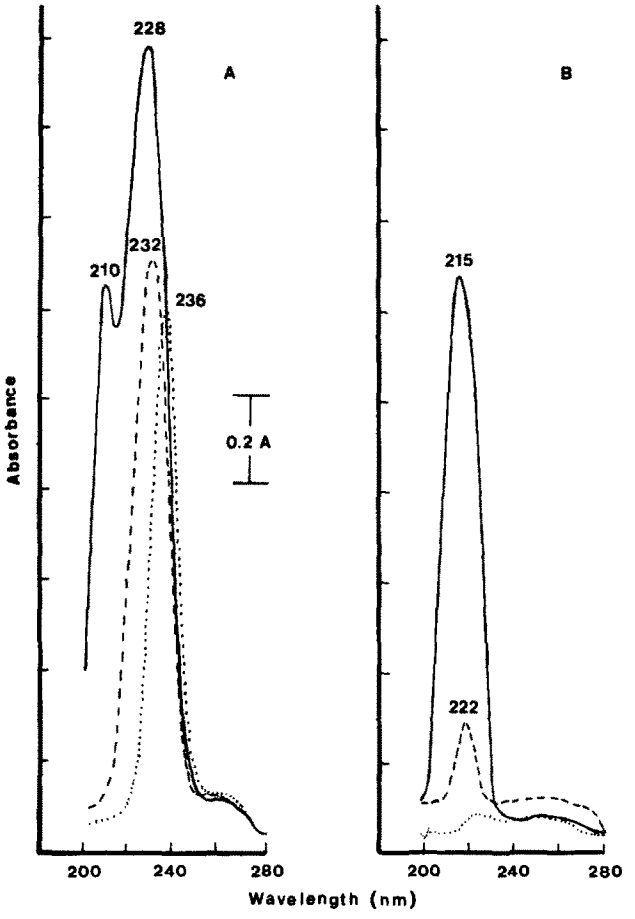

Fig. 2. Effect of protein concentration on the u.v. spectra of 5×10^{-5} M (A) TSO and (B) 1,2-diphenyl-1,2-ethanediol. Key: (——) phosphate buffer (pH 7.4, I = 0.2); (- - -) 0.25% (0.15 mg/ml) mouse cytosol; and (· · ·) 0.5% (0.30 mg/ml) mouse cytosol.

ml) used in this study. At higher concentrations this decrease might have to be taken into consideration, and the loss of TSO would have to be followed at a longer wavelength. As protein concentration increased, the λ_{max} of 1,2-diphenyl-1,2-ethanediol underwent a bathochromic shift into the 229 nm region; however, a large concomitant hypochromic effect obliterated any interference with TSO absorbance (Figs. 2B and 3B).

Hydration of TSO by the cytosolic epoxide hydrolase was measured by following the decrease in absorbance at 229 nm. A typical time course for the standard assay [0.25% (w/v) liver cytosolic enzyme solution, 5×10^{-5}M TSO] is shown in Fig. 4. The decrease in absorbance was linear for more than 45 min, and the initial velocity obtained for TSO hydration was comparable to the values determined by the GLC [6] and radiometric [8] point assay methods used in this laboratory. Nonenzymatic hydration of TSO in buffer at 37° was negligible. The u.v. spectrum of the reaction mixture after complete reaction was identical to the authentic *meso*-1,2-diphenyl-1,2-ethanediol (λ_{max} 221 nm, ε 6000 $M^{-1}cm^{-1}$) *in situ*. GLC analysis indicated that the cytosolic epoxide hydrolase, like the microsomal enzyme [18], hydrated TSO solely to the *meso* form and *cis*-stilbene oxide to the *threo* form. Optical activity was not determined for substrates or products.

Hydration of TSO by the crude cytosolic epoxide hydrolase was linear with protein in the range used in this study (0.02 to 0.63 mg/ml) and with time from 0 to > 35 min. Kinetic parameters, K_m and V_{max}, were determined by the double-reciprocal plot method of Lineweaver and Burk [19]. The hydration of TSO by this enzyme had an apparent K_m of 1.2×10^{-5} M and an apparent V_{max} of 5.9 nmoles/(min-mg protein) (Fig. 5) when cytosols from several mouse livers were examined.

Initial rates of reaction, in agreement with previously reported data [3, 20], indicated that cytosolic epoxide hydrolase activity was high in the control mouse and low in the rat. Induction with BHA caused a significant decrease in mouse cytosolic hydrolase activity (20% lower than control level) and an increase in glutathione epoxide transferase activity (8×). The initial rates of conjugation of TSO and GSH to form the thioether were very high in the rat and BHA-induced mouse and lower in the control mouse. The high levels of GSH transferase in the rat and BHA-induced mouse caused negligible interference with the epoxide hydrolase assay since

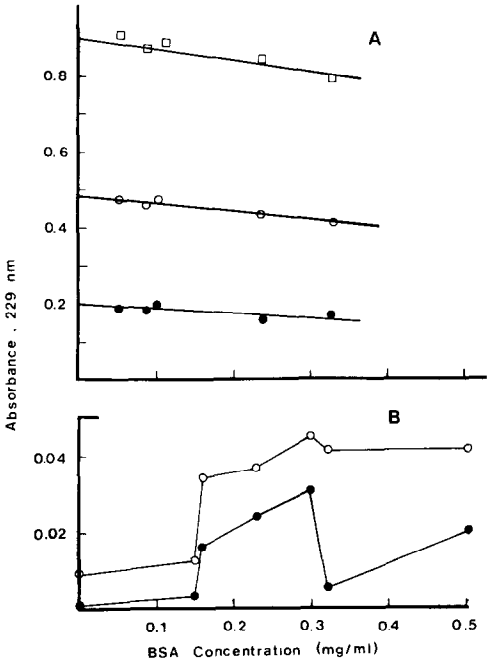

Fig. 3. Effect of protein (BSA) concentration on the absorbance of various concentrations of (A) TSO and (B) 1,2-diphenyl-1,2-ethanediol at 229 nm. Key: (□——□) 5×10^{-5} M, (○——○) 2.5×10^{-5} M, and (●——●) 1×10^{-5} M. The BSA solutions were made up in phosphate buffer (pH 7.4, I = 0.2).

GSH titers were normally low in the isolated cytosol due to disulfide formation during enzyme preparation. However, if the fractions are prepared at low pH, significant GSH can remain. Further depletion of GSH by the addition of 10 mM diethyl maleate caused no inhibition of the epoxide hydrolase activity when monitored spectrophotometrically or chromatographically.

DISCUSSION

A direct and continuous spectrophotometric assay for mammalian cytosolic epoxide hydrolase activity utilizing TSO as the substrate has been presented. The method is rapid, sensitive, highly reproducible, and has been in use in two laboratories for over a year. TSO at a concentration of 5×10^{-5} M has an absorption of approximately 1 absorbance unit, and this method readily allows the detection of an initial rate of hydration of 60 pmoles·min^{-1}·mg tissue equivalent^{-1} using rat cytosol (0.125% weight volume or ~ 0.1 mg protein/ml) which has very low epoxide hydrolase activity. This assay is as sensitive as, or approaches the sensitivity of the continuous assays developed for the microsomal epoxide hydrolase [21–25]. TSO is also nonvolatile and stable in an aqueous solution at 37° unlike the substrates used in some other epoxide hydrolase assays. Initial rates of hydration of TSO (5×10^{-5} M) by mouse liver cytosolic enzyme using the spectral assays are comparable to those obtained on several occasions by

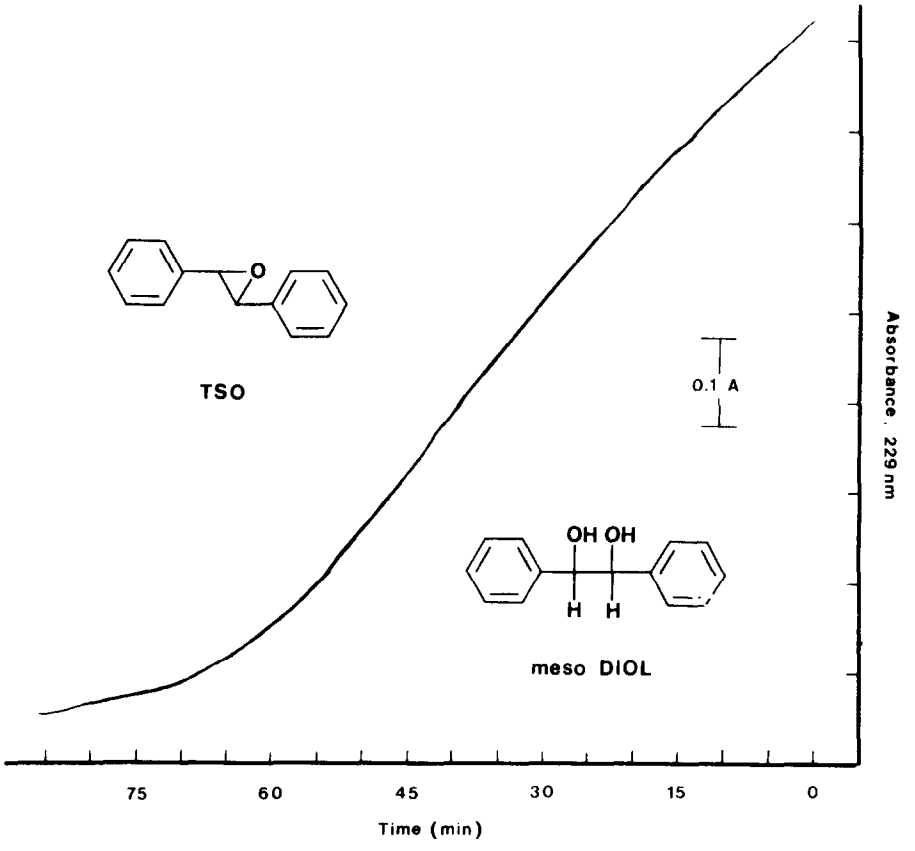

Fig. 4. Enzymatic hydration of TSO monitored at 229 nm. TSO (5×10^{-5} M) was incubated with 0.25% (0.15 mg/ml) mouse cytosol in phosphate buffer (pH 7.4, I = 0.2) at 37°.

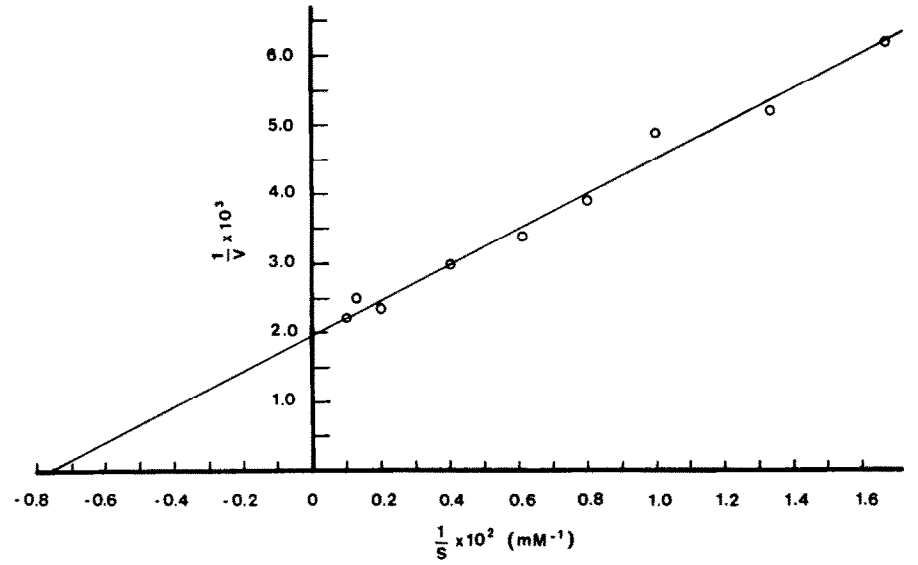

Fig. 5. Double-reciprocal plot for the hydration of TSO by crude mouse liver cytosolic epoxide hydrolase. Each initial velocity (V) is the mean of three determinations at two protein concentrations (0.15 and 0.33 mg/ml) each at nine substrate (S) concentrations (6×10^{-6} to 1×10^{-4} M). This experiment was repeated on several occasions with similar results. The line was fit to the experimental points by linear regression analysis with a correlation coefficeint of 0.993. An apparent K_m of 1.2×10^{-5} M and V_{max} of 5.9 nmoles·min^{-1}·(mg protein)$^{-1}$ were determined from this plot. These kinetic parameters agree closely with those found by another worker in this laboratory [K_m 1.1×10^{-5} M and V_{max} 6.9 nmoles·min^{-1}·(mg protein)$^{-1}$, C. M. Mullin and B. D. Hammock, manuscript submitted for publication].

the GLC and radiometric-TLC methods also used in this laboratory (374, 404 and 314 pmoles·min^{-1}·mg tissue equivalent^{-1}, respectively, for a single normal mouse cytosol). The K_m and V_{max} values derived for the hydration of TSO in mouse liver cytosol by this method are less than the respective values for *trans*-β-ethylstyrene oxide [8], indicating that *trans*-β-ethylstyrene oxide turns over faster and binds less tightly than TSO which is consistent with data from other substrate specificity and inhibitor studies ([2, 6], manuscript in preparation). The main advantage of this continuous and direct assay is that the determination of kinetic parameters is made facile relative to end-point assays. Initial rates, deviations from linearity, and variations in rates due to e.g. alterations of the reaction mixture are also rapidly and easily determined by this method. Many of the substrates discussed by Guengerich and Mason [22] are hydrolyzed by the cytosolic epoxide hydrolase, and this linked assay is applicable to a wider variety of structural types than the TSO assay. However, the TSO assay avoids numerous artifacts sometimes associated with "linked" assays, and in the species so far examined it is hydrated preferentially by the cytosolic-mitochondrial form of the enzyme. Although the techniques employed with TSO are not generally applicable to all structural types, the success of the TSO and other spectrophometric assays indicate that the techniques may be applied to a variety of compounds.

A major drawback is that the monitoring wavelength for the TSO assay is in an ultraviolet region where most aromatic compounds absorb strongly and thus difficulties in performing inhibitor screens with this assay may arise. Also, the low aqueous solubility of TSO (1×10^{-4} M) necessitates the use of a substrate concentration very near to the concentration needed for enzyme saturation as determined from the K_m. It is likely that both of these difficulties could be overcome by using substituted stilbene oxides as recently done for *p*-nitrostyrene oxide [25]. Although styrene oxide itself is an inhibitor of the cytosolic epoxide hydrolase at the millimolar levels commonly used for routine epoxide hydrolase analysis, it is rapidly hydrated by the cytosolic epoxide hydrolase of a variety of mammalian species at micromolar concentrations. Thus, *p*-nitrostyrene oxide and related compounds may also prove to be useful analytical tools. A second drawback of the assay is that the strong absorption of TSO at 210 nm precludes monitoring appearance of the diol at 215 nm. Possibly synthesis of the above derivatives will facilitate development of product appearance rather than substrate disappearance assays.

Glutathione epoxide transferase also may be measured using this assay since the resulting conjugate has an ultraviolet spectrum very similar to 1,2-diphenyl-1,2-ethanediol. However, because this method follows the loss of TSO, both conjugation and hydration are measured simultaneously. Therefore, the hydration rate must be subtracted to obtain the rate of glutathione conjugation to TSO in crude cytosol preparations. The results obtained indicate that TSO is a good substrate for glutathione epoxide transferase and that this assay could be applicable in a preparation where the transferase and hydrolase have been resolved. However, because of background hydrolysis and apparent interference at 229 by GSH itself, the sensitivity and reproducibility of

this assay method do not at this time equal that of the TLC [8], partition (S. S. Gill, manuscript in preparation), or spectrophotometric assays [26, 27] used in this laboratory to measure GSH-transferase activity.

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