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Radiometric Assays for Mammalian Epoxide Hydrolases and Glutathione S-Transferase

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A number of epoxides, including *cis*- and *trans*-stilbene oxides, were assayed as substrates for epoxide hydrolases (EHs) by gas–liquid chromatography. Radiolabeled stilbene oxides were prepared by sodium borotritide reduction of desyl chloride followed by ring closure with base treatment. Rapid radiometric assays for EHs were performed by differential partitioning of the epoxide into dodecane, while the product diol remained in the aqueous phase. Glutathione (GSH) transferase was similarly assayed by partitioning the epoxide and diol, if formed metabolically, into 1-hexanol, while the GSH conjugate was retained in the aqueous phase. The cytosolic EH rapidly hydrates the *trans* isomer while the *cis* is very poorly hydrated. In contrast, the *cis* is a better substrate for the microsomal EH than the *trans*. GSH transferase utilized both epoxides as substrates, but conjugation is faster with the *cis* isomer. Cytosolic EH activity is high in mouse but very low in rat and guinea pig. Microsomal EH activity, in contrast, is highest in guinea pig, intermediate in rat, and the lowest in mouse. GSH transferase activity, which is high in all three species, can be inhibited by chalcone, with an I_{50} of 3.1×10^{-5} M. These assays facilitate the rapid evaluation and direct comparison of epoxide-metabolizing systems in cell homogenates used in short-term mutagenicity assays, cell or organ culture, and possibly *in vivo*.

There has been intense interest in the metabolic fate of epoxidized xenobiotics due to the risks posed to human health by epoxides, both from natural and anthropogenic sources. Epoxides can also be formed *in vivo* in the metabolic conversion of olefinic precursors to hydrophilic compounds (1). A number of such epoxides, whether endogenously formed or from exogenous sources, are cytotoxic, carcinogenic, and/or mutagenic (2). In animal systems, such epoxides are degraded primarily by conjugation with glutathione (GSH),[2] a reaction catalyzed by GSH transferases (3), or hydrolyzed by epoxide hydrolases (EHs) to the corresponding 1,2-diols (1).

GSH transferase and EH activities have been monitored by a host of assay procedures. For example, GSH transferases are routinely monitored with chlorodinitrobenzene (4), benzo[*a*]pyrene 4,5-oxide, and styrene oxide (5,6); for additional assays see references in (3). EH activity in the microsomal and/or nuclear fractions is usually monitored with benzo[*a*]pyrene 4,5-oxide and styrene oxide (7–10), while activities in the cytosolic and mitochondrial fractions have been monitored with TESO, methyl epoxystearate, TSO, and an insect growth regulator (11–17). Thus, most of the assays utilize widely differing structures for the determination of the various epoxide-metabolizing enzymes, making comparative studies difficult. In addition, some of these assays are time consuming, often requiring separation of the product from the substrate by TLC or GLC or differential solvent extraction and purification prior to HPLC. The situation becomes still more complex if attempts are made to determine the relative rates of epoxide metabolism in crude tissue homogenates, cell or organ culture, or *in vivo*.

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[2] Abbreviations used: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; LSC, liquid scintillation counting; TSO, *trans*-stilbene oxide; CSO, *cis*-stilbene oxide; TESO, *trans*-β-ethylstyrene oxide; GSH, glutathione, EH, epoxide hydrolase.

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Based on a survey of the initial rates of hydration of a variety of epoxides by mouse cytosol and microsomes, we report below rapid and highly sensitive radiometric assays utilizing stilbene oxides as substrates for the microsomal and cytosolic EHs and GSH transferases.

METHODS

Synthesis of ^3H- and ^2H-labeled trans- and cis-stilbene oxides. To prevent initial decomposition of sodium borotritride, due to trace amounts of water and/or other impurities, a small quantity of sodium borohydride (J. T. Baker, 300 μg in 0.7 ml ethanol) was added to desyl chloride (95% pure, 108 mg, 0.45 mmol in 1 ml ethanol) and the reaction stirred at room temperature for 10 min. The reaction mixture and an ethanol wash (0.6 ml) was transferred to a vial on ice containing sodium borotritide (3.9 mg, 25 mCi, 246 mCi/mmol, New England Nuclear, Boston, Mass.) and stirred at ice temperature for 15 min and then at room temperature for 30 min. Subsequently, to get complete reduction of desyl chloride, sodium borohydride (3.9 mg in 0.5 ml ethanol) was added and the reaction mixture stirred for a further 20 min. After the resulting chlorohydrin was treated with 1 N NaOH (5 ml) at room temperature for 1 h, the mixture was extracted with 20% diethyl ether in hexane (2 ml $\times$ 3). The ether–hexane phases washed with saturated NaCl (2 ml) and dried with anhydrous Na_2SO_4 were combined and concentrated under N_2. The concentrate was applied to five TLC plates (silica gel GF_{254}, 0.25 mm, 20 $\times$ 20 cm, prewashed with methanol and dried) and developed in hexane: ether (10:1) three times; the first development was to 7 cm, the second to 12 cm, and the final to 18 cm. The bands, visualized under uv light and corresponding to CSO and TSO (R_f 0.3 and 0.4, respectively, in a single development in hexane–ether), were scraped, placed in sintered glass funnels, and eluted with ethanol. CSO and TSO were checked for radiochemical and geometrical purity by TLC in a number of solvent systems including hexane:ether (10:1) and tolune:propanol (19:1) and by GLC (1.5 mm $\times$ 1.5 m; 2.5% OV-101 on Gas Chrom Q, N_2 flow rate 20 ml/min). The uv spectra of the oxides were taken and an aliquot was counted to determine specific activity. The samples were diluted with hexane and sealed in glass ampoules which were subsequently stored at −20°C until required.

The ^2H-labeled stilbene oxides were similarly synthesized using $NaBD_4$ (8 mg) and desly chloride (108 mg, 0.45 mmol). The reaction mixture was worked up as described above and separated on preparative TLC plates (silica gel GF_{254}, 2.0 mm, 20 $\times$ 20 cm). The band corresponding to the combined ^2H-labeled stilbene oxides (*cis* and *trans*) was extracted to obtain 85 mg of ^2H-labeled stilbene oxides (96% yield, 95% pure by NMR, 57% *trans* and 43% *cis*, 99% incorporation of deuterium).

Synthesis of standard diols and GSH conjugate. To [^3H]CSO (7.0 mg, 11.8 mCi/mmol) in an ice bath, 0.05 N H_2SO_4 in 40% aqueous THF (0.6 ml) was added. The reaction mixture was then allowed to warm up with stirring to room temperature and was continued in the dark for 2 weeks. The reaction mixture was diluted with diethyl ether (1 ml), the ether phase separated, washed with saturated NaCl (2 $\times$ 2 ml), dried with anhydrous Na_2SO_4, and then evaporated under N_2. The residue dissolved in *n*-propanol (2 ml) was applied to a semi-preparative Spherosorb column (10 μm, 1.0 $\times$ 50 cm) and eluted with 1% methanol–1.5% *n*-propanol in 2,2,4-trimethylpentane. The *threo*- and *meso*-diols eluting at 67 and 73.5 min, respectively, were further purified on an analytical silica gel (5 μm, 0.2 $\times$ 25 cm) column and eluted with 1% methanol–1.5% propanol in 2,2,4-trimethylpentane. The *threo* and *meso* fractions, identified by the retention times of their respective *n*-butylboronic esters on GLC (2.5% OV-101 on Gas Chrom Q) using internal standards, were collected. Further identification was done by cochromatography with authentic standards and by uv.

Alternatively, the ^{3}H-labeled diols were prepared from [^{3}H]TSO and CSO using the cytosolic and microsomal EHs of guinea pig liver. The stilbene oxides (5×10^{-5} M) were incubated individually at 37°C in an Erlenmeyer flask containing a 6% w/v 10,000*g* supernatant of guinea pig liver. After a 2-h incubation (90 and 86% conversion of the *cis* and *trans,* respectively) the reaction mixture was extracted with petroleum ether (2×125 ml) and then with ether (2×125 ml). The ether phases were dried with anhydrous Na_2SO_4, concentrated under N_2, and diols separated by TLC using toluene–*n*-propanol (19:1).

To GSH (10^{-2} M) in distilled water (1 ml, pH adjusted to 9.5 with 1 N NaOH) was added [^{3}H]TSO (5×10^{-3} M) in 0.3 ml ethanol and the reaction stirred overnight at room temperature under a nitrogen atmosphere. The reaction mixture was extracted first with isooctane (2×1 ml), ether (1 ml), and then with 1-hexanol (1 ml). The aqueous phase was then spotted on Whatman chromatophates and developed in toluene–*n*-propanol (19:1). Bands of silica gel and cellulose were scraped and counted by LSC.

Partition characteristics. To determine the partitioning of stilbene oxide, stilbene diol, and the GSH conjugate of stilbene oxide, 5×10^{-5} M concentration (in 1 μl EtOH) of each, individually in sodium phosphate buffer (100 μl, pH 7.4, $I = 0.2$), unless otherwise mentioned, was partitioned against various solvents (200 μl). Aliquots of the aqueous and organic phases were removed by a Hamilton syringe and quantified by LSC.

Enzyme preparation. Livers of mice (Swiss–Webster, male, 28–30 g), rats (Sprague–Dawley, male, 180–220 g), and guinea pigs (Hartley, male, 330–380 g) were homogenized in 0.25 M sucrose and centrifuged at 10,000*g* for 10 min. The 10,000*g* supernatant fraction (S-10) was centrifuged at 100,000*g* for 60 min to give the microsomal pellet and the cytosolic supernatant. The microsomal pellet was resuspended in sodium phosphate buffer (pH 7.4, $I = 0.2$) and recentrifuged at 100,000*g* to give washed microsomes. Dilutions of the microsomal and cytosolic fractions were made in appropriate buffers prior to use. Protein levels were determined (18) with bovine serum albumin (fraction V) as standard.

Enzyme assays. Initial rates of microsomal and cytosolic hydration of unlabeled epoxide were determined in appropriate buffers (see below) by GLC. The epoxides (5×10^{-5} M) were incubated at 37°C for appropriate times in 1 ml of enzyme mixture, following which the reaction was terminated with approx 250 mg of NaCl. After the addition of an internal standard (an appropriate diol, 10^{-5} M, see Table 1), the enzyme mixture was extracted with ether (2×1 ml). The ether phases were dried over anhydrous Na_2SO_4, combined, and the ether then evaporated under a gentle stream of N_2. The residue derivatized by the addition of *n*-butylboronic acid (50 μg in 50 μl ethyl acetate) was then analyzed by GLC under conditions described in Table 1.

Enzyme assays using radiolabeled substrates were performed as follows. To either microsomal or cytosolic fractions (100 μl in 6×50-mm culture tubes) preincubated for 1 min at 37°C, TSO or CSO (5×10^{-5} M) was added in ethanol (1 μl). After incubation at 37°C for 10 min, the reactions were terminated by extracting the incubation mixture by rapid vortexing for 15–20 s with *n*-dodecane (200 μl). Following centrifugation, an aliquot (50 μl) of the aqueous phase was removed for quantitation by LSC. Enzymatic activity assays for microsomal and cytosolic EHs were performed at their optimal pH in Tris–HCl, pH 9.0, $I = 0.2$ buffer and sodium phosphate, pH 7.4, $I = 0.2$ buffer, respectively. GSH transferase activity was similarly monitored with TSO or CSO (5×10^{-5} M) in sodium phosphate, pH 7.4, $I = 0.2$ buffer with the addition of GSH (5 mM). The reaction was terminated by extracting with 1-hexanol (200 μl), centrifuged, and the aqueous phase quantified by LSC. Alternatively, EH and GSH transferase activities were measured by applying an aliquot (25 μl) of the aqueous phase

from an appropriate incubation to the loading zone of Whatman LK5DF silica-gel plates, developed in toluene:*n*-propanol (19:1) and assayed by LSC following procedures of Jerina *et al.* (7).

For determining the potency of various chalcones (1,3-diphenyl-2-propene-1-one and/or its substituted derivatives) as inhibitors of GSH transferase, the chalcones were added individually in 1 μl EtOH to a 1-min-preincubated mixture containing the cytosol (100 μl) and GSH (5 mM). After a further incubation of 1 min unless otherwise indicated, CSO (final concentration 5×10^{-5} M) in 1 μl EtOH was added. After incubation for 10 min, or as indicated otherwise, the reaction mixture was stopped and extracted with 1-hexanol as described above. Chalcones and chalcone oxides were synthesized as previously reported (19) or were commercially available.

RESULTS

The cytosolic EH of mouse liver rapidly hydrates a variety of *trans*-β-alkylstyrene oxides and TSO (Table 1) which are, however, poor substrates for the microsomal EH. Although TESO, previously radiosynthesized (12), is an excellent substrate, its use is limited by its high volatility at 37°C and slow nonenzymatic hydrolysis (12). In addition, the separation of *cis*- and *trans*-β-ethylstyrene oxides is tedious. The same limitations also apply to *trans*-β-propylstyrene oxide, although it is hydrolyzed faster than TESO by the cytosolic EH. In contrast, CSO and TSO are good substrates for the epoxide-metabolizing enzymes, have low volatility, and are stable to nonenzymatic hydration. The background rate remains essentially the same for incubations as long as a few hours when either

TABLE 1

RELATIVE HYDRATION RATES OF A VARIETY OF EPOXIDES BY MOUSE LIVER MICROSOMES AND CYTOSOL AS DETERMINED BY GAS–LIQUID CHROMATOGRAPHY

	GLC conditions[a]		Initial rates (nmol/min/mg protein[c])	
	Retention time of diol	Internal standard	Cytosol	Microsome
Trans-β-propylstyrene oxide	2.96	SD[b]	267.9	0.7
Trans-β-ethylstyrene oxide	2.30	SD	136.4	0.4
Trans-β-methylstyrene oxide	1.72	ABD	2.3	ND[d]
Cis-β-methylstyrene oxide	1.61	ABD	0.2	0.3
Styrene oxide	1.56	ABD	0.6	1.5
Allylbenzene oxide	2.12	SD	1.3	11.1
1-Phenoxy-2,3-epoxypropane	2.47	ABD	1.4	7.0
Trans-stilbene oxide	3.31	CPPD	8.4	ND
Cis-stilbene oxide	3.92	CPPD	0.9	4.1
1,2-Vinylcyclohexene oxide	1.54	ABD	ND	ND
7,8-Vinylcyclohexene oxide	1.54	ABD	0.7	2.3
1,2-Limonene oxide	2.27	SD	ND	10.6
7,8-Limonene oxide	2.27	SD	0.3	16.6

[a] Hewlett–Packard gas chromatograph with a 1.5 mm × 1.5-m, 2.5% OV-101 on Gas Chrom Q column and a N_2 flow rate of 20 ml/min was used. Column temperature was 170°C except for 1-phenoxy-2,3-epoxpropane and *trans*- and *cis*-stilbene oxides, which were run at 200°C.

[b] SD, styrene diol; CPPD, *p*-chlorophenoxypropane diol; ABD, allylbenzene diol.

[c] From Hammock and Hasagawa, in preparation.

[d] ND, not detectable.

TSO and CSO are incubated in phosphate buffer (pH 7.4, 5×10^{-5} M). The need for such stability and low volatility, as required in metabolic studies using cell culture, led us to choose TSO and CSO as obvious compounds to radiolabel from among a variety of compounds (Table 1). In addition, TSO and CSO can readily be labeled and subsequently separated by analytical TLC, HPLC, or GLC.

Mouse liver microsomal EH hydrolyzes a variety of aliphatic epoxides (Table 1). The most rapid hydrolysis is of 1,2- and 7,8-limonene oxides and allylbenzene oxide, which in contrast, are poor substrates for the cytosolic EH of mouse liver. These epoxides, particularly the limonene oxides, would thus be excellent substrates for monitoring microsomal EH in the presence of the cytosolic EH. However, CSO offers an advantage in that it can be readily radiolabeled and can be used in conjunction with TSO to monitor several reactions involving epoxide metabolism.

Sodium borotritide reduction of α-haloketones has previously been used for the synthesis of TESO (12). In a similar procedure, sodium borotritide reduction of desyl chloride followed by ring closure in base gave a high yield of TSO and CSO (*trans/cis* = 55/45) as determined by TLC, while with sodium borodeuteride a 57/43 ratio, by NMR, of *trans* to *cis* isomers was obtained. A radiochemical yield of >96% was obtained in the synthesis of ^{3}H-labeled stilbene oxides with a specific activity of 60 mCi/mmol for each of the two isomers. Purification by analytical TLC gave >99% pure stilbene oxides. Geometrical purity was >97% for both stilbene oxides as determined by TLC and GLC.

The widely differing octanol/water partition coefficients (log *P*) of stilbene oxides, stilbene glycol, and the GSH conjugate of stilbene oxide, 3.15, 1.49, and −3.54, respectively, were utilized in developing rapid partition assays for the cytosolic and microsomal EHs and GSH transferase. Table 2 shows that most solvents extract the stilbene

TABLE 2

PARTITIONING OF STILBENE OXIDE, STILBENE DIOL, OR THE GSH CONJUGATE OF STILBENE OXIDE BETWEEN VARIOUS ORGANIC SOLVENTS AND SODIUM PHOSPHATE BUFFER[a]

	Percentage epoxide into organic phase	Percentage diol into aqueous phase	Percentage diol into organic phase	Percentage GSH conjugate into aqueous phase
2,2,4-Trimethyl pentane	99.92	92.98		100.00
Undecane	99.93	92.18		—
Dodecane	99.91	91.98[a]		99.99
Cyclohexane	99.75	85.23		—
Toluene	99.87	17.24		—
Xylene	99.98	25.88		99.86
p-Cymene	99.98	43.28		—
Isopropylxylene	99.99	28.70		
2,6-Dimethyl-4-heptanone	99.80	9.15		
1-Butanol	—	—	97.78	—
1-Pentanol	—	—	97.98	—
1-Hexanol	99.89	—	98.11	99.64
1-Heptanol	—	—	97.84	—
1-Octanol	—	—	97.05	99.98
1-Decanol	—	—	96.53	—
Ethylacetate	—	—	—	100.00
Diethyl ether	—	—	—	100.00

[a] The balance of the epoxide, diol or GSH conjugate partitions into the second phase. Thus, with dodecane, 91.98% of stilbene diol will partition into the aqueous phase, while 8.02% will partition into the organic phase.

oxide; however, most of these solvents with the exception of saturated alkanes also extracted large amounts of stilbene diol from the aqueous phase. Thus, for EH assays, epoxide and diol were differentially partitioned by vigorous vortexing of the incubation mixture with dodecane. A correction was made for the 8% extraction of the diol into the organic phase. No significant changes in the partitioning characteristics of the epoxide and diol were observed with an increase in protein concentration to 1 mg/ml, change in buffers (Tris, phosphate, glycine, or acetate), salt concentration (to 1 M NaCl), or the addition of detergent (1% Brij 35, 0.2% Lubrol PX). Differential partitioning of the epoxide and diol is improved by adding methanol into the aqueous phase. With 30% methanol only 3.5% of the diol partitions into the organic phase, while no change was observed in the partitioning characteristics of the epoxide. However, addition of higher levels of methanol resulted in increased partitioning of the diol into the organic phase. Addition of methanol could thus serve to stop the enzyme reaction more rapidly and result in a more efficient partition assay. Hexane and other low-boiling alkanes can also be used to differentially extract the epoxide, and an amount of diol similar to that reported for dodecane was extracted. However, quantification of the organic phase, if necessary, is more difficult than for dodecane due to the volatility of hexane.

Both the epoxide and the diol partition into the organic phase when a series of alcohols is used (Table 2). In contrast, little if any of the GSH conjugate was extracted with these solvents. Thus, for GSH transferase assays, the GSH conjugate of the epoxide was differentially partitioned from the epoxide and the enzymatically formed diol, by extracting the incubation mixture with 1-hexanol. Little epoxide (<0.2%) or diol (<2%) was left in the aqueous phase.

Stilbene oxides, selective substrates for the microsomal and cytosolic EHs, are also good substrates for GSH transferase. In the cytosolic fraction TSO is hydrolyzed more rapidly

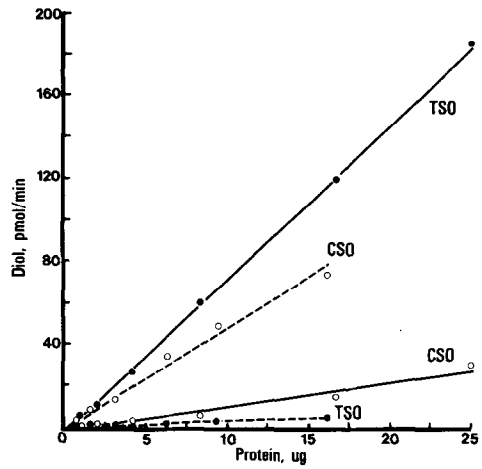

FIG. 1. Rate of hydration of TSO and CSO with varying levels of the cytosolic (● —— ●) and microsomal (O - - - O) fractions of mouse liver. Cytosolic and microsomal epoxide hydrolase assays were performed at pH 7.4 and 9.0, respectively.

than CSO at pH 7.4, while CSO is hydrolyzed more rapidly than TSO by the microsomal fraction at pH 9.0 (Fig. 1). CSO is conjugated more rapidly by GSH in the presence of the cytosolic GSH transferase than is TSO (Fig. 2). In the absence of GSH transferase, however, spontaneous reaction of GSH is faster with TSO than with CSO. Thus, with these differences in selectivity and the ability to separate microsomes and cytosol by differential

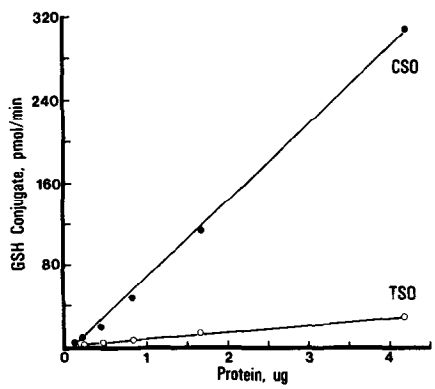

FIG. 2. Rate of GSH conjugation of TSO and CSO with varying levels of the cytosolic fraction of mouse liver at pH 7.4.

centrifugation, selective assays were developed as follows. Microsomal EH was monitored at pH 9.0 with microsomes and CSO as substrate, cytosolic EH was monitored at pH 7.4 with the cytosol and TSO, and GSH transferase activity was monitored with the cytosol and CSO at pH 7.4. Dodecane and hexanol were used as the extracting solvents for EH and GSH transferase assays, respectively.

Utilizing these substrates and incubation conditions, the levels of the microsomal and cytosolic EHs and GSH transferase were established in three mammalian species. The guinea pig has the highest level of GSH transferase and microsomal EH, while the mouse has the highest level of cytosolic EH (Table 3). These data confirm previous reports which showed the guinea pig and mouse to have high microsomal and cytosolic EH levels, respectively (20–22). The relative activities of GSH transferase in the three species studied, however, differ from published reports (3), possibly due to differences in substrates used for the assay. TSO has previously been demonstrated to undergo GSH conjugation catalyzed by sheep liver GSH transferase, but at a slower rate (23). In guinea pig liver S-10 fraction, TSO is converted to the *meso* diol while CSO forms the *threo* diol indicating *trans* hydration. In addition, in no case was *cis* hydration observed with any of the substrates examined in Table 1 with either mouse microsomes or cytosol.

These partition assays can also be utilized to determine microsomal and cytosolic EH and GSH transferase activities in crude liver homogenates or S-10 fractions. For example, in crude mouse liver homogenates the activities of CSO hydrolase at pH 9.0, TSO hydrolase at pH 7.4, and CSO GSH transferase at pH 7.4 were 0.22, 0.29, and 2.68 nmol min^{-1} mg tissue eq^{-1}, respectively. In contrast, the CSO microsomal EH, TSO cytosolic EH, and CSO cytosolic GSH transferase activities in mouse liver are 0.08, 0.31, and 3.08 nmol min^{-1} mg tissue eq^{-1}, respectively. Thus, an approximate indication of the relative levels of epoxide-metabolizing enzymes in mouse

TABLE 3

MICROSOMAL AND CYTOSOLIC EPOXIDE HYDROLASE AND CYTOSOLIC GLUTATHIONE TRANSFERASE ACTIVITIES IN THREE MAMMALIAN SPECIES[a]

Species	Microsomal epoxide hydrolase				Cytosolic epoxide hydrolase				Cytosolic glutathione transferase	
	CSO		TSO		CSO		TSO			
	7.4	9.0	7.4	9.0	7.4	9.0	7.4	9.0	CSO	TSO
Mouse	3.2 ± 0.1	5.3 ± 0.2	1.1 ± 0.04	0.3 ± 0.02	1.2 ± 0.01	0.5 ± 0.03	7.3 ± 0.2	3.4 ± 0.1	73.7 ± 0.8	6.9 ± 1.4
Rat	6.0 ± 0.04	11.1 ± 1.4	0.1 ± 0.1	0.1 ± 0.1	0.05 ± 0.04	0.04 ± 0.01	0.06 ± 0.01	0.09 ± 0.01	83.4 ± 10.1	21.3 ± 1.6
Guinea pig	3.7 ± 0.3	63.5 ± 7.9	0.2 ± 0.04	4.8 ± 2.9	0.1 ± 0.01	0.2 ± 0.01	0.5 ± 0.02	0.2 ± 0.02	203.2 ± 5.0	16.9 ± 4.2

[a] Rates given are in nmol min^{-1} mg $protein^{-1}$. Rates obtained by the partition assay for the mouse were closely related to those obtained by the TLC assay.

liver can be obtained with crude homogenates.

With mouse liver cytosol the partition assay for cytosolic EH can be run without much interference from endogenous GSH. In rat and guinea pig, however, endogenous GSH can interfere with the cytosolic EH assay. The endogenous GSH can be removed by dialyzing the cytosol, passing it through G-25, depleting the GSH, or by alternatively accounting for a higher background rate using the GSH transferase assay as control. When GSH transferase activity is high, however, the best success has usually been achieved by dialysis of the cytosol.

The microsomal and cytosolic EHs can be inhibited by trichloropropene oxide and chalcone oxides, respectively (1,19). For these compounds to be used as inhibitors in cell culture and *in vivo,* it is critical to know their effects on other epoxide-metabolizing enzymes, in particular GSH transferase. Thus, chalcones and chalcone oxides were screened to determine their effect on GSH transferases. Because chalcone oxides are much less soluble in ethanol than are the chalcones, all assays were performed at 10^{-4} M for chalcones and 10^{-5} M for the chalcone oxides. Of the chalcones used, the greatest inhibition was observed with chalcone, with an I_{50} of 3.1×10^{-5} M (Table 4). A number of other chalcones were also quite potent inhibitors. The chalcone oxides, in contrast, showed little inhibition at the levels used.

Since chalcones exhibit α,β-unsaturation, attempts were made to determine whether the inhibition observed is due solely to GSH depletion. Addition of an extra 5 mM of GSH to the incubation restored only 9.1% of the inhibited GSH transferase activity. Further, there was no difference in inhibition of enzyme activity whether GSH was added before or after the addition of chalcone. The possibility of chalcone reacting with GSH to form an active inhibitory product was checked by preincubating GSH with chalcone for varying periods of time before the addition of substrate and enzyme. No significant changes were observed between preincubation times of 0 to 10 min. Increasing GSH and enzyme preincubation times without chalcone addition increased the rate of GSH conjugation (10-min preincubation, 30% increase). Under similar conditions but with the addition of chalcone, no change in inhibition was observed. However, increasing the preincubation time of chalcone and the enzyme increases the inhibition observed even when GSH is added just before the addition of CSO.

TABLE 4

INHIBITION OF THE CYTOSOLIC GSH TRANSFERASE BY VARIOUS CHALCONES AND CHALCONE OXIDES[a]

Compound	Percentage inhibition at 1×10^{-4} M (chalcone)	Percentage inhibition at 1×10^{-5} M[b] (chalcone oxide)
—	0	0
Chalcone	83.3 (3.1×10^{-5} M)[c]	1.8
4-Fluoro	71.3	2.4
4'-Fluoro	62.0	−1.2
4-Bromo	77.5	11.9
4'-Bromo	58.7	1.2
4-Hydroxy	49.0	—
4'-Hydroxy	63.6	—
4-Isopropyl	81.8	10.1
4-*n*-Butyl	63.3	12.5
4-*n*-Octyl	2.5	9.6
4-Acetamido	68.3	—
4'-Acetamido	43.3	—
4-Phenyl	25.0	13.2
4'-Phenyl	24.2	12.0
4-Methoxy	35.8	14.4
4'-Methoxy	28.3	9.0
4-Methyl	42.6	12.5
4'-Methyl	7.8	5.4
4-Nitro	19.4	16.2
4'-Nitro	23.3	5.4
4-Benzyloxy	24.0	17.4
4-Benzyloxy-4'-chloro	24.8	—
4-Benzyloxy-4'-Methyl	9.3	—
4-Dimethylacetamido	48.8	—
4-Dimethylacetamido-4'-Bromo	50.4	—
4-Phenoxy	—	12.0

[a] Assayed with CSO as substrate.
[b] Synthesized by C. Mullin.
[c] I_{50} for chalcone.

DISCUSSION

The procedure used in the radiolabeling of TSO and CSO illustrates a facile synthetic route for radiolabeling various epoxides which may serve as potential substrates for EHs and GSH transferases. Such a route has previously been used for the synthesis of TESO, a substrate for the cytosolic EH. It is further possible with these procedures to radiolabel epoxides with extremely high specific activities. Thus, TSO and CSO have been synthesized with carrier-free sodium borotritide, resulting in specific activities of 10 Ci/mmol. In addition, carrier-free *trans*- and *cis*-β-methyl-, β-ethyl, and β-propylstyrene oxides have been synthesized by similar procedures (Hammock *et al.*, unpublished).

The partition assays developed here and elsewhere (11,12) show the potential of using differential extraction to monitor EH and GSH transferase activity. In fact, using sequential extraction, it is possible to monitor both EH and GSH transferase activity in the same enzyme reaction. Data from such experiments will rapidly indicate the relative enzyme activities of the competing pathways, and such data may prove valuable in analyzing the metabolic capability of S-10 preparation or cell lines used in short-term mutagenicity assays. The ability to utilize sequential extraction proved particularly useful in the analysis of epoxide, diol, and epoxide conjugates in the culture media of primary hepatocytes (Gill *et al.*, unpublished).

TSO and CSO also represent one of a few substrates with widely differing affinity for enzymes with changes in geometrical isomers. The *cis* isomer is a much better substrate for the mouse, rat, or guinea pig microsomal EH than for the cytosolic EH. In contrast, the *trans* isomer, as reported earlier (15), is hydrolyzed very slowly by the microsomes and rapidly by the cytosolic fraction. Both these compounds are good substrates for the GSH transferase, with CSO conjugation being more rapid than TSO conjugation. Nonenzymatic conjugation, however, is significant with TSO, and negligible with CSO. These substrates may prove useful as probes to determine the relative importance of the various epoxide metabolism pathways particularly in cell cultures and in animals *in vivo* where cellular integrity is maintained. The similarity in chemical structure and lipophilicity makes CSO and TSO a particularly useful pair of substrates for such comparison. Preliminary results with primary hepatocyte cultures show that these substrates indeed undergo different routes of metabolism.

The chalcones were found to be better inhibitors of GSH transferase than cytosolic EH, while the reverse is true with chalcone oxides (19). In contrast, the microsomal EH is stimulated by both chalcone and chalcone oxide (24). The best inhibitor of GSH transferase is the unsubstituted chalcone. The chalcone, at the levels used in this paper, apparently acts directly on GSH transferase; however, it is possible that at higher concentrations the chalcone, an α,β-unsaturated compound, can be a GSH depleter.

Thus, selective substrates and selective inhibitors may be important tools in unraveling the complexities of epoxide metabolism. For example, the chalcones may prove particularly useful in inhibiting the conjugation of GSH with leukotriene A, which results in the formation of leukotriene C (slow-reacting substance) (25), both *in vitro* and *in vivo* and thereby determines the effects of varying leukotriene C levels. The chalcone oxides may similarly prove valuable in inhibiting the hydrolysis of leukotriene A to leukotriene B (26), particularly if the cytosolic epoxide hydrolase catalyzes this reaction.

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