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Metabolism of Tridiphane (2-(3,5-Dichlorophenyl)-2(2,2,2-trichloroethyl)oxirane) by Hepatic Epoxide Hydrolases and Glutathione *S*-Transferases in Mouse[1]

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Metabolism of Tridiphane (2-(3,5-Dichlorophenyl)-2(2,2,2-trichlorethyl)oxirane) by Hepatic Epoxide Hydrolases and Glutathione *S*-Transferases in Mouse. MAGDALOU, J., AND HAMMOCK, B. D. (1987). *Toxicol Appl. Pharmacol.* **91,** 439–449. The transformation of the herbicide tridiphane (Tandem, Dowco 356, 2-(3,5-dichlorophenyl)-2(2,2,2-trichloroethyl)oxirane by the epoxide-metabolizing enzymes, epoxide hydrolases (EH) and glutathione *S*-transferases (GST), was investigated in mouse liver microsomes and cytosol. The microsomal EH catalyzed the formation of tridiphane diol. The production of this metabolite was prevented by cyclohexene oxide at 1 mM, a known inhibitor of microsomal EH. The structure of the diol was verified by comparison of retention time or R_f of the compound with those of an authentic standard using gas–liquid chromatography or thin-layer chromatography techniques. The diol formed a diester with 1-butane boronic acid or an aldehyde with lead tetraacetate. Mass spectral analysis supported the structural assignment. After optimization of the assay conditions, kinetic constants for the hydration of tridiphane by the microsomal EH were determined (K_m = 65 μM and V_{max} = 0.9 nmol/min/mg protein). Dietary exposure of mice to the hypolipidemic drug clofibrate at a dose of 0.5% (w/w) for 2 weeks increased by 173% the metabolism of tridiphane to tridiphane diol by the microsomal fraction. No diol could be detected following incubation of tridiphane with the cytosolic EH, even after induction by clofibrate. Tridiphane was also a substrate for GST, but administration of clofibrate did not change the specific activity for the formation of the glutathione conjugate. The herbicide was a rather weak inhibitor of the microsomal EH and the cytosolic GST activities measured with *cis*-stilbene oxide and *trans*-stilbene oxide as substrates with I_{50}'s of 3.0×10^{-5} and 1.8×10^{-4} M, respectively. Tridiphane diol was a poor inhibitor of the enzymes studied, and the glutathione conjugate of tridiphane caused marked inhibition of only the GST activity (I_{50}, 2.0×10^{-5} M). By contrast the activity of cytosolic EH (*trans*-stilbene oxide) was relatively insensitive to the addition of tridiphane or of tridiphane metabolites.

Tridiphane is a newly introduced herbicide which prevents grass proliferation in crops (Zorner and Olson, 1981) and selectively synergizes atrazine toxicity in several plants. This effect can be related to its transformation into a glutathione conjugate by glutathione *S*-transferases (GST, EC 2.5.1.18). The conjugate as well as tridiphane itself is able to inhibit atrazine detoxication, and this inhibition could explain the herbicidal activity of tridiphane (Lamoureux and Rusness, 1986). The metabolism of tridiphane has been most studied in plants and little is reported about its effects on other living systems, particularly

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in mammals; however, tridiphane recently has been shown to inhibit the metabolism of diazinon in house flies and to synergize its toxicity (Lamoureux and Rusness, 1987). Recently Moody and Hammock (1987) demonstrated the ability of this compound to induce some enzymes in mice. Administration of tridiphane increased the liver weight and the microsomal protein content. It also induced the enzymes involved in epoxide metabolism including epoxide hydrolases (EH, EC 3.3.2.3) and GST. Only the enzyme responsible for the transformation of cholesterol epoxide was decreased. Interestingly, like the hypolipidemic drug clofibrate, tridiphane is a peroxisome proliferator.

As an oxirane-containing structure, it is reasonable that tridiphane could be metabolized by EH and GST. In this respect, Lamoureux and Rusness (1986) have reported that the cytosolic GST from housefly and equine liver catalyzed conjugation of tridiphane with glutathione. Four different EHs have been characterized in mammals. Two of them, a microsomal cholesterol EH (Levin *et al.*, 1983; Sevanian and McLeod, 1986) and a leukotriene A4 EH (McGee and Fitzpatrick, 1985), have been recently described. They appear to be involved largely in the metabolism of endogenous epoxides. Two other EHs, located in cytosol (Hammock *et al.*, 1976; Gill and Hammock, 1980) and in microsomes (Oesch, 1973) catalyze the hydration of a large variety of endogenous and xenobiotic epoxides, and their substrate specificity has been extensively studied (Oesch *et al.*, 1971, 1984). The microsomal EH hydrates many epoxides on cyclic systems as well as mono-1,1-disubstituted, and *cis*-1,2-disubstituted epoxides. By contrast, many aliphatic epoxides including the *trans*-1,2-disubstituted epoxides are among the best substrates tested for the cytosolic EH (Hammock and Hasagawa, 1983; Mumby and Hammock, 1979). Although the activity in microsomal EH is enhanced by several drugs including phenobarbital (Bresnick *et al.*, 1977) and clofibrate (Fournel *et al.*, 1987), the cytosolic EH appears only induced by peroxisome proliferators such as clofibrate or tridiphane (Hammock and Ota, 1983; Loury *et al.*, 1985). On the other hand, GSTs constitute a family of isozymes mainly present in cytosol (Jakoby, 1978). Most GST isozymes appear to not be induced by clofibrate (Hammock and Ota, 1983; Loury *et al.*, 1985; Moody *et al.*, 1985), but their activity is weakly enhanced or unchanged after tridiphane treatment, depending on the type of substrate used (Moody and Hammock, 1987).

The purpose of this work is to determine the transformation pathways of tridiphane in mouse liver via the epoxide-metabolizing enzymes and the effect of clofibrate on the metabolism of the herbicide. The inhibitory potency of tridiphane itself and of the corresponding metabolites on the activity of these enzymes also will be reported.

METHODS

Materials. Tridiphane, tridiphane diol, and 2-(3,5-dichlorophenyl-uL-[^{14}C])-2(2,2,2-trichloroethyl)oxirane, ([^{14}C]tridiphane) (14 mCi/mmol) were provided by Dow Chemical Co. (Midland, MI). The radiochemical purity was estimated to be 94.2%. Clofibrate was kindly supplied by Ayerst Laboratories (New York, NY). *trans*-Stilbene oxide (TSO), *cis*-stilbene, cyclohexene oxide, and 1-butane boronic acid were purchased from Aldrich (Milwaukee, WI). *cis*-Stilbene oxide (CSO) and tritiated CSO and TSO were prepared according to Gill *et al.* (1983). Reduced glutathione and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). The Bio-Rad protein dye reagent and the ACS scintillation cocktail were from Bio-Rad Laboratories (Richmond, CA) and Amersham (Arlington Heights, IL), respectively. All the solvents were of the best purity commercially available.

Animals and enzyme preparation. Male C57 black mice, 25–30 g (Simonsen Labs, Gilroy, CA) were housed in steel cages with kiln-dried pine shavings as bedding in an environmentally controlled room (12 hr light cycle, 22.5–24.0°C, constant humidity). They were fed a Purina rodent chow and had tap water *ad libitum*. A group of mice was fed the chow containing 0.5% (w/w) clofibrate for 2 weeks in order to induce the microsomal and the cytosolic EH. After 12-hr fasting, the mice were killed by cervical dislocation. Their livers were removed and

perfused with ice-cold 1.15% KCl (w/v). The livers were washed with 100 mM potassium phosphate buffer pH 7.4, dried, and finally weighed. They were then homogenized in this buffer with a Polytron apparatus (Brinkmann Instruments, Westbury, NY) for 20 sec to give a 10% (of original liver weight) homogenate. The homogenate was centrifuged for 10 min at 10,000*g* (Sorvall Instrument, DuPont, Wilmington, DE) and the supernatant was decanted and centrifuged again for 60 min at 100,000*g* in a L3-40 Beckman ultracentrifuge (Beckman Instrument, Palo Alto, CA). The decanted supernatant constituted the cytosolic fraction. The microsomal pellet was washed, resuspended in the phosphate buffer, and collected again by centrifugation 60 min at 100,000*g*. All the centrifugations were run at 4°C. After homogenization, the microsomal fraction and the cytosol were stored at −80°C. Their protein content was determined by Bradford's assay (Bradford, 1976) modified for an enzyme-linked immunosorbent assay reader with computer readout as previously described (Moody *et al.*, 1985).

Metabolism of tridiphane. Enzyme incubations were conducted in glass tubes. When tridiphane was considered as substrate for the microsomal and the cytosolic EH, the assays were carried out as follows: in 100 μl diluted microsomes or cytosol previously equilibrated for 1 min at 37°C, [^{14}C]tridiphane in 2 μl ethanol (1–50 nmol, 8000 cpm) was added. The pH of the reaction was 9.0 (100 mM Tris–HCl) and 7.4 (76 mM Na/K phosphate buffer) when the activity of the microsomal and cytosolic EH were respectively measured. After incubation for various times (5 to 60 min), the reaction was stopped by addition of about 250 mg NaCl and placement in an ice bath. The sample was extracted twice with 1 ml ether and the organic phase was dried over anhydrous Na_2SO_4. The ether phase was evaporated under a gentle stream of nitrogen and the residue was finally dissolved in 50 μl ethanol. Under our conditions, ether extracted more than 97% of the tridiphane and corresponding radioactive metabolites. The reaction products were separated by thin-layer chromatography (TLC) on silica gel LK5DF plates (250-μm thickness, Whatman, Clifton, NJ) using hexane–ethyl acetate 3:1 (v/v) as the mobile phase. The position of the radioactive materials was determined by a Bioscan TLC scanner equipped with an Auto Changer 3000 and a System 2000 imaging scanner (Bioscan, Inc., Washington, DC). The apparatus was coupled to an Apple IIc computer for data processing. After the plates were scraped, the product was finally quantitated by liquid scintillation counting. Control experiments consisted of assays without enzyme or with 10-min boiled subcellular fraction. When significant, the enzymatic rates were corrected for control. To test the hypothesis that the Bioscan TLC scanner could be used quantitatively, the percentage conversion of tridiphane to metabolite was first determined by scanning and then by scraping and liquid scintillation counting. The results of extensive comparisons were compared by linear regression.

Formation of the glutathione conjugate of tridiphane was measured essentially as suggested by Wixtrom and Hammock (1985). Tridiphane (10 nmol) and radiolabeled [^{14}C]tridiphane (8000 cpm) were added to the dilute cytosol in 76 mM phosphate buffer, pH 7.4, and immediately prior to assay 500 nmol reduced glutathione in buffer was added. The mixture was incubated at 37°C and then the reaction was stopped by the addition of 200 μl *n*-hexyl alcohol. After the emulsion was shaken and broken by centrifugation, aliquots of the aqueous phase were taken for scintillation counting.

Enzyme assays and enzyme inhibition. Microsomal EH and cytosolic EH and GST activities toward TSO and CSO were measured using the radiometric partition assays described by Hammock *et al.* (1985). They were expressed as specific activity (nmol/min/mg protein). In order to determine if tridiphane and its corresponding metabolites tridiphane diol and glutathione conjugate were able to inhibit these epoxide-metabolizing enzymes, the putative inhibitor (10^{-7} to 10^{-3} M) was added in 1 μl ethanol (tridiphane diol) or water (glutathione conjugate) 2 min before the addition of the substrate to the enzyme fraction. The mixture was gently vortexed and incubated at 37°C for 10 min. The enzyme assay was carried out as previously indicated by Hammock *et al.* (1985). Controls without inhibitors were always run and were used in calculations to represent 100% enzyme activity. I_{50} values were determined using the linear portion of the inhibition curve and calculated by least-squares regression analysis. In some experiments, cyclohexene oxide, a known inhibitor of the microsomal EH (Oesch *et al.*, 1971, 1978; Levin *et al.*, 1978), was also used.

Determination of apparent K_m and V_{max}. The K_m and V_{max} of the hydratation of tridiphane by microsomal EH were calculated from the Michaelis–Menten equation using double reciprocal plots. The best fit line was obtained by regression analysis.

Identification and verification of the chemical structure of tridiphane diol. The metabolite of tridiphane was biosynthetized on a large scale. Diluted microsomes (8 ml, 2.4 mg protein/ml) in 100 mM Tris–HCl buffer (pH 9.0) were incubated at 37°C for 90 min with 1.6 μmol tridiphane and [^{14}C]tridiphane (0.3 μCi) in 160 μl ethanol. The extraction conditions were the same as those previously described. After separation of the diol from tridiphane by TLC and detection of the radioactive material, the radioactive zone containing the product was scraped and extensively washed with methanol and the silica was pelleted by centrifugation. The methanolic solution of putative tridiphane diol was concentrated by gentle evaporation of the solvent under nitrogen.

Attempts to synthesize the diol from tridiphane by acid hydrolysis were not successful. These attempts consisted of dissolving various concentrations of ^{14}C-labeled

herbicide in 40% aqueous tetrahydrofuran or 40% aqueous dioxane containing H_2SO_4 (0.05 N). The mixtures were agitated at room temperature or refluxed at 100°C for 24 hr. After addition of NaCl to saturation and neutralization of the aqueous phase with NaOH, the mixtures were extensively extracted with ether. The ether phase was dried over Na_2SO_4 and evaporated to dryness. This residue was examined by TLC with detection by uv, selective spray reagents, and radioactivity as well as by GLC. Under these conditions no diol could be found and the starting material was recovered unchanged. Thus, subsequent studies were based on a synthetic diol standard provided by Dow Chemical. In this case the diol was prepared by reaction of tridiphane with sodium acetate in the presence of acetic acid to yield the corresponding 2-ol, 3-acetate which was then hydrolyzed in aqueous base. This diol also was used as a possible inhibitor of EH and GST and as a standard for identification of the microsomal metabolite as described below.

The chemical structure of the metabolite formed by the liver microsomal EH was verified by co-chromatography with the tridiphane diol standard using gas–liquid chromatography and TLC. Gas chromatography was performed on a Varian-Aerograph (series 1400) gas–liquid chromatograph with a flame ionization detector. The silanized glass column (1.5 m × 1.5 mm, i.d.) was packed with 3.0% OV 225 (Supelco, Inc., Bellefonte, PA) on Supelcoport (100/120 mesh). The injection port, detector, and column temperatures were 250, 270, and 220°C respectively. Nitrogen (carrier gas), hydrogen, and air flow rates were 15, 20 and 300 ml/min, respectively. The ability of the diol to form a less polar diester in the presence of 1-butane boronic acid or an aldehyde with lead tetraacetate was also checked. For these investigations, the radiolabeled metabolite was mixed with unlabeled diol standard and was taken to dryness. Then 50 μl of 1-butane boronic acid (10 mg/ml in ethyl acetate) or lead tetraacetate (5 mg/ml in benzene) was added. After the complexes were mixed, they were immediately chromatographed on silica gel LK5DF plates using hexane–ethyl acetate 3:1 (v/v) as the mobile phase. The products were visualized either by iodine vapor exposure, by autoradiography (X-ray film, Kodak, Rochester, NY), or by a Bioscan TLC scanner. Both derivatives presented a R_f significantly higher than that of the starting diol with the cold standard co-migrating with the labeled metabolite in each case (see Results).

Finally the structure of tridiphane diol formed by microsomal EH was verified by gas chromatography–mass spectrometry (GC–MS) and comparison with the authentic standard. For this purpose the sample was injected (on-column injection) in the GC part of a ZAB-HS-2F (VG Analytical, Wythenshawe, UK) equipped with a 30-m DB-5 capillary column (J & W Scientific, Inc., Rancho Cordova, CA). The GC oven was programmed at 180–260°C (6°C/min) with a helium carrier gas flowing at 1 ml/min at 180°C. The mass spectra were obtained with an electron ionization of 20 eV.

Synthesis of glutathione conjugate of tridiphane. Tridiphane (300 nmol), [^{14}C]tridiphane (0.40 μCi), and reduced glutathione (1.6 mmol) were added to 100 ml of dimethylformamide containing 5% (v/v) triethylamine. The mixture was stirred under nitrogen atmosphere for 36 hr at room temperature. After addition of chloroform and water, the glutathione conjugate was extracted in the aqueous phase which was thereafter concentrated by evaporation to dryness by vacuum centrifuge. The final product was fully water soluble. Thin-layer chromatography of this compound in the previous developing solvent system hexane–ethyl acetate 3:1 (v/v) and scanning of the plate revealed a single ^{14}C-labeled spot, ninhydrin positive, which did not migrate from origin. When *n*-butanol–acetic acid–water 12:3:5 (v/v) was used as the developing solvent system the product presented a R_f value of 0.55 (see Results). A similar R_f was obtained when the glutathione conjugate of tridiphane biosynthesized by GST was chromatographed under the same experimental conditions. The structure of the synthetic glutathione conjugate of tridiphane was verified by fast atom bombardment (FAB) mass spectrometry, positive ion, on a ZAB-HS-2F mass spectrometer (VG Analytical) with a 8-keV xenon atom beam. The sample was mixed in a matrix of glycerol containing 1% (v/v) trifluoracetic acid. The mass fragmentation pattern was similar to that reported by other authors (Lamoureux and Rusness, 1986). The glutathione conjugate was quantitated by liquid scintillation counting and tested as inhibitor of epoxide-metabolizing enzymes.

Statistics. The significance between clofibrate-treated and control mice was determined by the Student *t* test for small samples and nonpaired series. A difference between groups of $p < 0.05$ was considered significant. Data from the TLC scanning and from liquid scintillation counting were compared by linear regression.

RESULTS

The formation of a polar metabolite after incubation of tridiphane with the microsomal EH was followed by TLC. Under conditions where tridiphane alone presented a single peak with an R_f of 0.86, after incubation with the microsomal enzyme a second sharp peak of R_f 0.32 appeared. The formation of this more polar product was totally prevented when boiled microsomes were used as the enzyme source or when cyclohexene oxide (1 mM), a selective inhibitor of microsomal EH, was added in the incubation

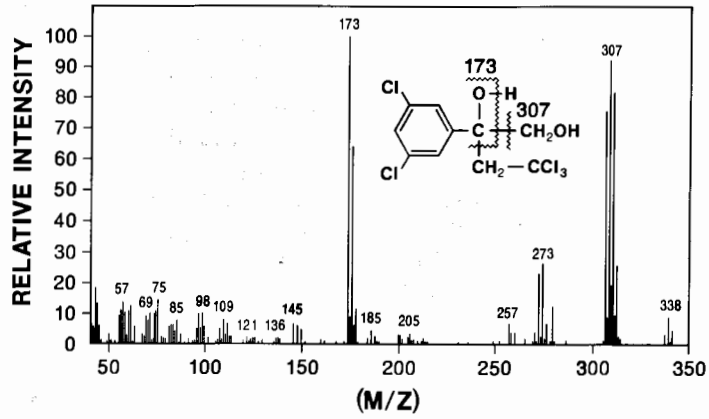

FIG. 1. Mass spectrum of tridiphane diol. Tridiphane was incubated with microsomes, extracted, and analyzed by GC–MS according to the conditions described under Materials and Methods. The fragmentation pattern of the metabolite is also given. The part of the spectrum between m/z 950 and 350 is expanded seven times.

mixture prior to the addition of tridiphane. No such peak was observed when tridiphane was incubated with cytosol from nontreated or clofibrate-treated mice under conditions where TSO was rapidly hydrated. Following partial purification of the metabolite by TLC, liquid scintillation counting indicated that greater than 95% of the radioactivity co-chromatographed with the authentic diol standard. For further support of the metabolite's structure, microchemical derivatization was used. Reaction of the radioactive metabolite and unlabeled standard gave a diester with an R_f(0.60) higher than that of the starting diol. A similar pattern was obtained when lead tetraacetate was used to oxidize the diol to the corresponding aldehyde. Following chemical modification, greater than 95% of the radioactivity of the metabolite then co-chromatographed with the higher R_f derivative in each case.

Figure 1 shows the mass fragmentation pattern of the diol after incubation of tridiphane with microsomes. The spectrum was characterized essentially by three ion clusters, the molecular ion (m/z 338), $C_6H_3Cl_2$-C^+-(OH)-CH_2-CCl_3 (m/z 307), and $C_6H_3Cl_2$-C-O^+ (m/z 173). This mass profile matched precisely with that of the synthetic diol. The FAB mass spectrum of the glutathione conjugate of tridiphane was similar to that published previously (Lamoureux and Rusness, 1986).

In order to determine if the Bioscan TLC scanner was useful in monitoring and quantitating the formation of tridiphane diol, we compared the ratios of diol to epoxide obtained after scanning the plate (time of exposure, 15 to 30 min) with those measured after scraping the plate and liquid scintillation counting. A reasonably good correlation (r^2, 0.79; slope, 0.86) was found between the two techniques. However, when the amount of diol was less than 7% the correlation was poor. The optimization of formation of diol is shown in Fig. 2. The reaction was linear as a function of time up to 60 min (Fig. 2A) and as a function of the protein concentration to 90 μg of protein per assay (Fig. 2B). Results were similar whether scanning or scintillation counting was used to generate the data. Under assay conditions which gave a linear response as a function of time and protein content and conformed to Michaelis–Menton assumptions, a K_m of 65 μM and V_{max} of 0.9 nmol/min/mg protein were obtained (Figs. 3A and 3B).

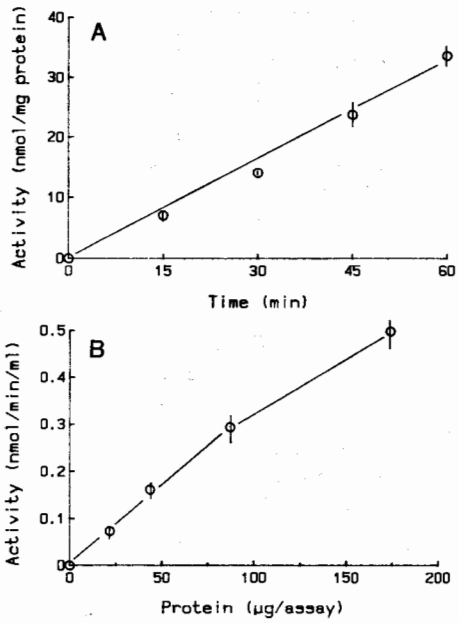

FIG. 2. Optimization of the production of tridiphane diol by mouse liver microsomes. (A) Linearity of the reaction as a function of time; (B) linearity as a function of protein content. Each point corresponds to mean ± SD of, at least, three independent determinations.

The formation of the glutathione–tridiphane conjugate by cytosolic GST was also investigated (Fig. 4). Under the assay conditions used, the reaction was linear up to 15 min and up to 80 μg per assay (Figs. 4A and 4B), respectively. However, we were unable to meet conditions required for Michaelis–Menton kinetics to determine valid K_m's and V_{max}'s of the reaction with glutathione.

Table 1 indicates the effect of dietary clofibrate on the hydration of tridiphane and formation of its glutathione conjugate. For comparison the action of clofibrate on the EH and GST activities using the standard substrates CSO and TSO is also mentioned. Clofibrate increased 1.8 and 3.1 times the activity of the two enzyme activities when measured with CSO and TSO, respectively. The hypolipidemic drug enhanced by 2.7 times the formation of tridiphane diol catalyzed by the microsomal EH. By contrast, the cytosolic EH was ineffective in the production of this diol even after induction of the enzyme by clofibrate (Table 1). The hypolipidemic drug had an opposite effect on GST activity when measured with TSO (Table 1). The activity was decreased by 50% over the controls but no significant change in the formation of tridiphane conjugate was observed after treatment by clofibrate.

The inhibitory potency of tridiphane, tridiphane diol, and glutathione conjugate of tridiphane on the activities of EH and cytosolic EH and GST is indicated in Fig. 5. Tridiphane decreased the activities of microsomal EH (I_{50}, 3.0×10^{-5} M) (Fig. 5A) and GST (I_{50}, 1.8×10^{-4} M) (Fig. 5C). Cytosolic EH was only slightly affected either by tridiphane or by the two metabolites (Fig. 5B). Tridiphane diol had a less inhibitory effect on microsomal EH (I_{50}, 3.0×10^{-4} M) than tridiphane itself (Fig. 5A); it did not inhibit the activity

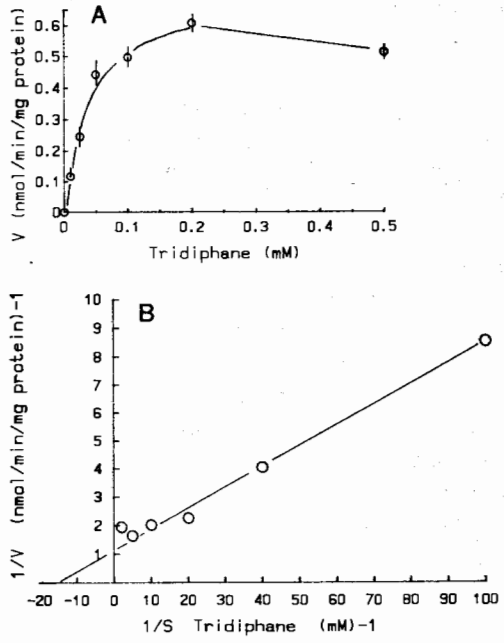

FIG. 3. Determination of the kinetic constants of microsomal epoxide hydrolase for tridiphane. (A) Velocity of the reaction; (B) double reciprocal plot representation. Each point represents the mean ± SD of three independent determinations.

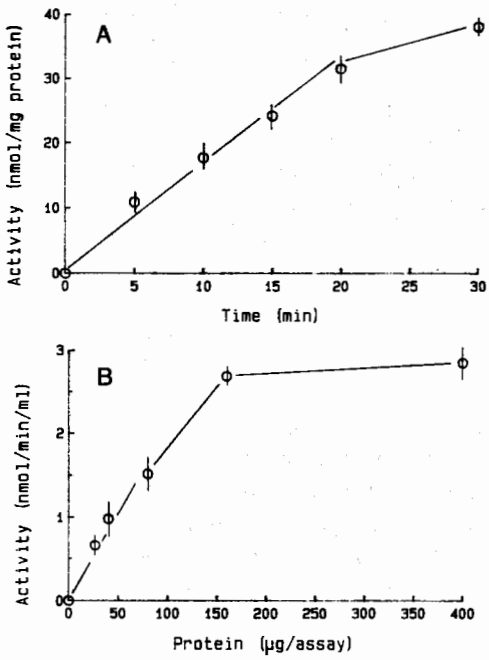

FIG. 4. Production of tridiphane–glutathione conjugate by glutathione *S*-transferase in mouse cytosol. (A) Linearity of the reaction as a function of time; (B) linearity as a function of protein content. Each point corresponds to mean ± SD of, at least, three independent determinations.

of GST (Fig. 5C). Finally, the glutathione conjugate was a good inhibitor of GST activity (I_{50}, 2.0×10^{-5} M) (Fig. 5C) while having minimal effect on the other activities monitored.

DISCUSSION

This study clearly shows that tridiphane can be metabolized *in vitro* by the microsomal EH and not the cytosolic form of enzyme from mouse liver. The herbicide belongs to the class of 1,1-disubstituted epoxides which are mainly hydrated by the microsomal EH (Oesch *et al.*, 1971; Hammock and Hasagawa, 1983). This membrane-bound enzyme is more generally involved in the hydration of lipophilic epoxides (Oesch, 1974), including polycyclic epoxides such as benzo[*a*]pyrene-4,5-oxide (Schmassmann *et al.*, 1976). Lipophilicity seems to play a large role in the transformation of epoxides by the microsomal EH. Oesch (1974) pointed out that mono-substituted oxiranes with a lipophilic substituent larger than an ethyl group readily interact with the lipophilic binding site of the enzyme protein. The low water solubility of tridiphane (Lamoureux and Rusness, 1986) emphasizes its transformation by the microsomal EH. Finally, the use of cyclohexene oxide, a selective inhibitor of microsomal EH (Levin *et al.*, 1978), which stopped the formation of tridi-

TABLE I

EFFECT OF CLOFIBRATE ON THE HYDRATATION AND GLUTATHIONE CONJUGATION OF STANDARD SUBSTRATES AND TRIDIPHANE BY MOUSE LIVER EPOXIDE-METABOLIZING ENZYMES

	Microsomal EH (nmol/min/mg)		Cytosolic EH (nmol/min/mg)		GST (nmol/min/mg)	
Treatment	CSO	Tridiphane	TSO	Tridiphane	TSO	Tridiphane
Control	4.16 ± 0.75	0.41 ± 0.08	7.24 ± 0.63	ND[a]	13.75 ± 2.77	2.05 ± 0.38
Clofibrate	7.53 ± 0.92[b]	1.12 ± 0.20[b]	22.5 ± 1.2[b]	ND[a]	6.95 ± 1.03[b]	2.48 ± 0.68
	(1.81)	(2.73)	(3.10)		(0.50)	(1.21)

Note. Clofibrate was given in the diet at a dose of 0.5% (w/w) for 2 weeks. Values are means ± SD of at least three independent experiments. Values in parentheses are the ratio of clofibrate-treated animals to controls.

[a] ND, not detectable. The limit of detection of the diol formation was 0.05 nmol/min/mg protein.

[b] Significantly different from control, $p < 0.05$.

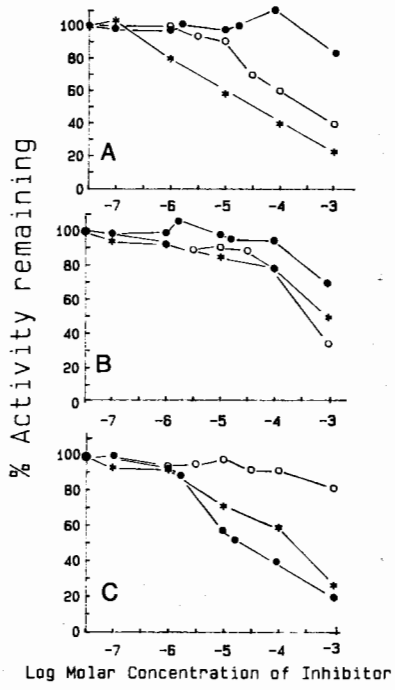

FIG. 5. Inhibition of epoxide-metabolizing enzymes by tridiphane (* — *), tridiphane diol (○ — ○), or glutathione conjugate of tridiphane (● — ●). The inhibitors (10^{-7}–10^{-3} M) were added in the incubation medium 2 min before addition of CSO for measurement of microsomal epoxide hydrolase (A) or of TSO for measurement of cytosolic epoxide hydrolase (B) or glutathione *S*-transferase (C). Each point represents the percentage of the activity remaining; 100% is taken as the control value obtained without any inhibitor. Note that the higher concentrations of tridiphane used exceed its water solubility.

phane diol, is also an argument in favor of the hydration of the herbicide by this type of EH. On the other hand, the lack of hydration of tridiphane by the cytosolic EH is consistent with previous information on this enzyme (Hammock and Hasagawa, 1983; Mumby and Hammock, 1975; Gill and Hammock, 1979).

When rate of hydration of tridiphane is compared to commonly used substrates of the microsomal EH such as CSO or benzo[*a*]-pyrene-4,5-oxide, tridiphane could not be considered as a good substrate. The K_m for the hydration of the herbicide (65 μM) was in the same range as that of the benzo[*a*]pyrene-4,5-oxide (Mukhtar *et al.*, 1979); however, the rate of the reaction (less than 1 nmol/min/mg protein) was much slower. This could be due to the presence of the bulky 3,5-dichlorophenyl and the trichloroethyl moieties which cause steric hindrance at the potentially reactive benzyl carbon or more likely due to the electron withdrawing properties of both the 3,5-dichlorophenyl ring and the dichloroethyl substituent. Tridiphane is remarkably stable in acidic conditions in that no spontaneous hydrolysis of the epoxide moiety could be detected when the herbicide was refluxed in mineral acid. Tridiphane also was very stable in a variety of buffer systems. For instance, no hydrolysis of the epoxide was detected following very long periods of incubation in 100 mM Tris–HCl buffer (pH 9.0), indicating that the microsomal EH is absolutely essential for the production of the diol metabolite. Although tridiphane is volatile in the field, its low volatility during incubation and workup compared to model substrates like styrene oxide, its very low rate of spontaneous hydrolysis, and the selectivity of hydration by the microsomal EH indicate that tridiphane could be a useful diagnostic substrate for investigating epoxide hydration.

In contrast to older proportional flow TLC scanners which are slow and generally yield qualitative data, our results with tridiphane indicate that enzyme assays can be based on separation by TLC followed by rapid quantitative analysis using a positron scanner. Such an approach offers advantages with regard to laboratory safety and speed of analysis by eliminating scraping of TLC plates. Even initial rates could be determined by using higher levels of radioactivity and an internal radioactive standard, while the ability to monitor both the glutathione conjugate and the diol in a single scan offers an advantage over partition methods.

Oesch *et al.* (1971) reported that 1,1-disubstituted oxiranes are generally less active as enzyme inhibitors on the microsomal EH

than oxiranes with a 1-aryl substituent (styrene oxide) or with a 1-alkyl substituent (1-octene oxide). Thus, the weak inhibition observed with tridiphane could be anticipated from the literature. Interestingly, only the microsomal EH and the cytosolic GST activities were affected by micromolar levels of the herbicide. This observation indicates that tridiphane has some affinity for the two enzyme systems. The inhibitory potency was far less than that reported for 4′-phenylchalcone oxide, 1,1,1-trichloropropane oxide, or 2-bromo 4′-phenylchalcone, which inhibit the cytosolic, microsomal EH and GST, respectively (Hammock *et al.*, 1986; Miyamoto *et al.*, 1987). As with most diols previously studied, tridiphane diol exhibited only marginal activity on the enzymes studied.

Like equine liver, housefly, or plants (Lamoureux and Rusness, 1986), mouse liver GST catalyzed conjugation of tridiphane with glutathione. The R_f value of the glutathione conjugate after TLC was in good agreement with that reported by these authors using a similar solvent system. However, the herbicide can be considered as only a moderate substrate for the GST because the specific activity obtained with the herbicide was much lower than that reported with CSO, TSO, or other compounds routinely used to measure the GST activity (Moody and Hammock, 1987). Indeed, GSTs constitute a family of enzymes with broad substrate specificity (Jakoby, 1978; Chasseaud, 1979). The formation of the glutathione conjugate can be considered as an effective pathway for transformation of tridiphane into a water-soluble metabolite. However, the glutathione conjugate of tridiphane exhibited a rather specific inhibitory effect on GST activity which was more powerful than that of tridiphane itself. Lamoureux and Rusness (1986) reported that, in plants, this metabolite decreased a large variety of GST activities measured with several substrates; moreover, the I_{50} value for the inhibition of GST by the glutathione conjugate of tridiphane in corn was of the same order as that observed in this work with the GST from mouse liver which hydrates TSO. It seems that the metabolism of tridiphane by GST could influence the elimination of other drugs or chemicals that are conjugated with glutathione and therefore modify their toxicity.

Clofibrate administration for 2 weeks in mice increased the production of tridiphane diol by the microsomal EH, but did not change that of the glutathione conjugate. This hypolipidemic drug induces a large variety of enzymes (Lundgren *et al.*, 1987; Schladt *et al.*, 1987) and, of special note, the cytosolic EH (Moody *et al.*, 1985). Only a limited number of xenobiotics, all of which are peroxisome proliferators, are able to increase cytosolic EH activity (Pichare and Gill, 1985). By contrast, the microsomal EH is enhanced by various chemicals including clofibrate (Oesch *et al.*, 1973; Wixtrom and Hammock, 1985; Fournel *et al.*, 1987). Clofibrate enhanced the activity of the microsomal EH toward CSO (this report; Moody and Hammock, 1986), although to a lesser extent when compared to the induction of cytosolic EH. The increase in the production of tridiphane diol as the result of microsomal EH induction followed the same trend. By contrast, clofibrate lowered the activity of GST monitored with TSO. This decrease has already been reported (Moody *et al.*, 1985; Moody and Hammock, 1987) when TSO or 3,4-dichloronitrobenzene are used as substrates to monitor the enzyme activity; on the other hand, the activity toward CSO, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, or 1-chloro-2,4-dinitrobenzene was not affected. In fact, Loury *et al.* (1985) report a significant induction of GST activity by clofibrate when CSO is used as a substrate. With the protocol used here, clofibrate did not change the production of tridiphane conjugate. The difference in the ratio of induction after clofibrate treatment could suggest that conjugation of TSO and tridiphane to glutathione is catalyzed by two different isozymes.

In conclusion, tridiphane is metabolized, *in vitro,* by both microsomal EH and cytosolic GST in subcellular fractions of mouse liver. Since it is not detectably metabolized by the cytosolic EH, the compound may have utility as a diagnostic substrate. Because Moody and Hammock (1987) have shown that tridiphane, like clofibrate, induces the microsomal EH, tridiphane falls into the interesting group of xenobiotics that induce their own degradation. It should be noted, however, that the formation of the glutathione conjugate of tridiphane was not induced by clofibrate. Certainly more studies are needed to determine the metabolic fate of this herbicide *in vivo.*

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