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Purification of Microsomal Epoxide Hydrolase from Liver of Rhesus Monkey: Partial Separation of *cis*- and *trans*-Stibene Oxide Hydrolase[1]

DAVID E. MOODY[2] AND BRUCE D. HAMMOCK[3]

Departments of Entomology and Environmental Toxicology, University of California, Davis, California 95616

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Solubilized rhesus monkey liver microsomes were used as the starting material for the purification of epoxide (*cis*-stilbene oxide) hydrolase. Successive chromatography over DEAE-Sephacel followed by CM-cellulose resulted in two peaks of activity, CM A and CM B. Passage of these two eluates over separate hydroxyapatite columns resulted in two peaks of activity from CM A, HA A1, and HA A2, and one peak from CM B and HA B, with respective recoveries of 1, 7, and 0.2% of *cis*-stilbene oxide hydrolase activities. A similar recovery was found for benzo[*a*]pyrene-4,5-oxide hydrolase, while *trans*-stilbene oxide hydrolase activity coeluted only in HA A2. Fraction HA A1 was homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Immunoblots of the three eluates and solubilized microsomes incubated with anti-HA A1 demonstrated a single band at 49 kDa in each fraction. The three eluates were differentially affected by the inhibitors of epoxide hydrolase, trichloropropene oxide and 4-phenylchalcone oxide, and addition of Lubrol PX and phospholipid. Immunoprecipitation of HA A2 resulted in coprecipitation of *cis*- and *trans*-stilbene oxide hydrolase activity. Upon immunoprecipitation of solubilized microsomes, all the *cis*-stilbene oxide and benzo[*a*]pyrene-4,5-oxide, but only 50–60% of *trans*-stilbene oxide hydrolase activity was precipitated. These studies support findings with other species that (i) an immunochemically distinct cytosolic-like epoxide hydrolase exists in microsomes, and (ii) microsomal epoxide hydrolase activity can be separated during ion-exchange chromatography giving proteins with similar molecular weights and immunochemical cross-reactivity. The precipitation of *cis*- and *trans*-stilbene oxide hydrolase activity in eluate HA A2 provides convincing evidence that these isozymes are not structurally identical.

Epoxides (arene oxides, oxiranes) are often unstable and have been implicated as the reactive functional group on several carcinogens, mutagens, and cytotoxic compounds (1–3). A primary route of biodegradation of epoxides occurs from their hydrolysis to diols (dihydrodiols) by enzymes referred to as epoxide hydrolases (EC 3.3.2.3) (4–7). In mammalian tissue there is now evidence for four distinct epoxide hydrolases. Two, a microsomal cholesterol epoxide hydrolase (8, 9) and a cytosolic leukotriene epoxide hydrolase (10), appear to have limited substrate-specificity. The other two, however, accept a broad number of endogenous and xenobiotic compounds as substrates. One, microsomal epoxide hydrolase, is located primarily in the microsomal cell fraction with lesser activity in nuclear membranes and cytosol (4–6), while the other, cytosolic epoxide hydro-

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[2] Current address: Center for Human Toxicology, University of Utah, Salt Lake City, UT 84112.

[3] To whom correspondence should be addressed.

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lase, is primarily located in the cytosol cell fraction with activity also found in mitochondria, microsomes, and peroxisomes (7, 11–13). The relative abundance of these two epoxide hydrolases in cellular fractions of tissue may play an important role in the metabolic fate of epoxides.

The existence of the cytosolic-like epoxide hydrolase in mouse liver microsomes has been confirmed by immunochemical criteria (12). For other species, including human and non-human primates, less stringent evidence exists for the microsomal localization to two xenobiotic-metabolizing epoxide hydrolases. The specificity of *trans*-stilbene oxide (TSO)[4] for the cytosolic-like epoxide hydrolase (14–16) has proven useful for these studies when compared to substrates specific for the microsomal epoxide hydrolase, such as benzo[*a*]pyrene-4,5-oxide or *cis*-stilbene oxide. In rats and mice microsomal TSO hydrolase responds differently to chemical induction (17–19), and in human microsomes as well, the two activities have unique inhibition profiles (19, 20). Both activities have been found in hepatic microsomes from rhesus monkey (21, 22).

The microsomal epoxide hydrolase has been purified from microsomes of rodent (23–27) and human (26–29), but not non-human primate, livers. Under certain ion-exchange chromatographic conditions, the activity can be separated into different fractions (23, 25, 26). It has not yet been ascertained with certainty whether these fractions of epoxide hydrolase activity represent distinct isozymes or the same isozyme with minor differences in microenvironment or post-translational modifications. Furthermore, in none of these studies was the fate of the cytosolic-like epoxide hydrolase in microsomes considered during the purification.

Non-human primates are often a valuable model for human disease. For the study of toxic responses it is important to know if these model systems contain a similar array of xenobiotic-metabolizing enzymes. In several cases, this has proven to be true, with the enzyme content not only quantitatively similar to humans, but also with the enzymes sharing a strong structural relatedness. Rhesus monkey liver has now been used as a source of microsomes to purify epoxide hyrolase activity. A purification protocol previously found to separate epoxide hydrolase activity (26) was employed, and the relative recovery of the microsomal- and cytosolic-like epoxide hydrolases was monitored by substrate and immunochemical specificity.

EXPERIMENTAL PROCEDURES

Materials. All materials, unless specified otherwise, were purchased from commercial suppliers at the highest purity available. The specific reagents for the experiments described were Lubrol PX, 1,1,1-trichloropropane oxide (TCPO), and egg yolk phosphatidylcholine (Sigma Chemical Co., St. Louis, MO); CM 52-cellulose and LK5DF silica gel TLC plates (Whatman Chemical Separation, Clifton, NJ); isooctane, TSO, and *cis*-stilbene (Aldrich Chemical Co., Milwaukee, WI); hydroxyapatite (Bio-Gel HPT), nitrocellulose membranes, goat anti-rabbit IgG coupled to immunobeads, goat anti-rabbit IgG conjugated to horseradish peroxidase, and 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, CA); and DEAE-Sephacel (Pharmacia Fine Chemicals, Upsala, Sweden). CSO, [^{3}H]CSO, [^{3}H]TSO (15,16), and 4-phenylchalcone oxide (4-PCO) (30) were synthesized as previously described. Unlabeled and [^{3}H]BPO were generously provided by Dr. Richard Armstrong (Dept. of Chemistry, Univ. of Maryland) and Dr. Michael McManus (then at NIH) respectively.

Preparation of microsomes. Slices of liver were obtained from two untreated male Rhesus monkeys at the California Primate Research Center. Liver sections were removed and processed immediately. Livers were placed in ice-cold 76 mM sodium phosphate (pH 7.4), minced, homogenized (Polytron, several 20-s pulses interspersed with 40 s cooling), and brought to a volume of three times the wet weight. The homogenates were then centrifuged for 20 min at 10,000*g*, the supernatant was collected and centrifuged for 60 min at 105,000*g* and the pellets were resuspended in 50 mM Tris (pH 7.4), 125 mM KCl and recentrifuged with the resulting pellets resuspended in the phosphate buffer, collected as microsomes, and stored at −80°C.

Solubilization of microsomes. Prior to solubilization the microsomes were resedimented and resuspended

[4] Abbreviations used CSO, *cis*-stilbene oxide; TSO, *trans*-stilbene oxide; BPO, benzo[*a*]pyrene-4,5-oxide; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCPO, 1,1,1-trichloropropene oxide; 4-PCP, 4-phenylchalcone oxide.

in 0.2 M potassium phosphate (pH 7.4), 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF to a concentration of 2.5 mg protein/ml. Twenty percent Lubrol PX in the same buffer was then added to the microsomes as they were being stirred in an ice bath. As described by Guengerich *et al.* (26), Lubrol PX was added over a period of 30 min to a final concentration of 1% and stirring was continued for a total of 60 min. The solution was then centrifuged for 60 min at 105,000*g* and the supernatant was collected as solubilized microsomes and stored at −80°C.

Purification of epoxide hydrolase. Epoxide hydrolase was purified from solubilized hepatic microsomes using a modification of the procedure described by Guengerich *et al.* (26) for rat liver epoxide hydrolase (Fig. 1). All procedures were carried out at 4°C and all buffers contained potassium phosphate (at the molarity and pH specified) and 0.05% Lubrol PX. Solubilized microsomes (1–1.2 g protein) were dialyzed for 12 h against 15 vol of 5 mM (pH 7.25) buffer and then loaded onto a 2.5 × 30-cm column of DEAE-Sephacel equilibrated with the same buffer. The column was then washed with 500 ml of the same buffer and eluted with a 800-ml gradient of 5 to 500 mM (pH 7.25) buffer. A single peak of activity was detected and fractions with the highest activity were pooled.

The DEAE-Sephacel eluate was dialyzed for 12 h against 15 vol of 5 mM (pH 6.25) buffer and then loaded onto a 2.5 × 30-cm CM 52-cellulose column equilibrated with the same buffer, washed with 400 ml of the same buffer, and eluted with a 700-ml gradient of 5 to 500 mM (pH 6.25) buffer with two peaks (CM A and CM B) of activity eluting. Complete elution of the two peaks occurred at a salt concentration of approximately 320 mM, as is noted in the protocol described (Fig. 1).

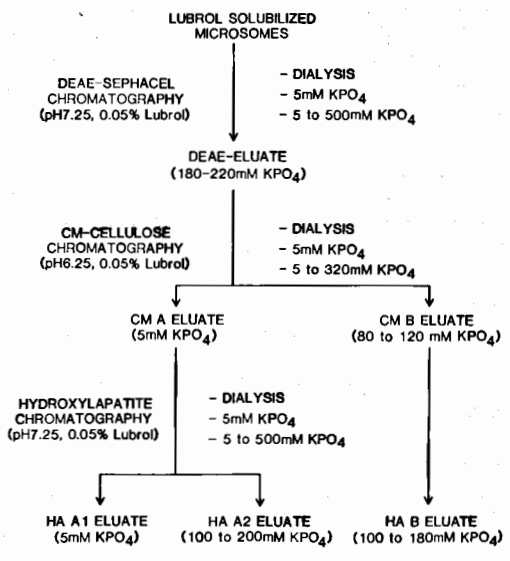

FIG. 1. Protocol for the purification of epoxide hydrolase from Lubrol PX-solubilized Rhesus monkey liver microsomes.

The peak fractions from CM A and CM B were pooled separately and each was dialyzed against 10 vol of 5 mM (pH 7.25) buffer for 12 h. They were then loaded onto two similar 2.5 × 12-cm hydroxyapatite columns equilibrated with the same buffer and each column was washed with 400 ml of the same buffer. The columns were then eluted with 450 ml/column gradients of 5 to 500 mM (pH 7.25) buffer. Two peaks were collected from column A (HA A1 and HA A2) and one from column B (HA B).

Biochemical assays. Epoxide hydrolase activity was monitored using tritiated CSO, TSO, and BPO as substrates. The tritiated stilbene oxides were greater than 98% radiometrically pure and 99% geometrically pure, with specific activities of 69 mCi/mmol, and used at 5×10^{-5} M. Diol formation from the stilbene oxides was estimated in isooctane extracts of the incubation media as previously described (15). To initiate the reaction, 100 μl of protein diluted in 76 mM sodium phosphate buffer (pH 7.4) was preincubated at 37°C for 30 s and then 1 μl of substrate (ca. 20,000 cpm) dissolved in ethanol was added using a repeating Hamilton syringe. The mixtures were then incubated in a shaking water bath for 20–60 min, and then the reaction was terminated by addition of 200 μl isooctane. After vortexing for 30 s, the phases were separated by centrifugation, and a 50-μl aliquot of the aqueous phase was collected for determination of diol content by liquid scintillation counting. Controls were routinely run with substrate added to buffer only, and were used to estimate background radiation and nonenzymatic hydrolysis. The coefficient of variation for triplicate samples was approximately 5%, so that assays with cpm less than 10% greater than background controls were considered nonsignificant. Assays were routinely performed at two or more dilutions to assure linearity with protein added. Unless specified otherwise, CSO hydrolysis was conducted at pH 9.0 and TSO hydrolysis at pH 7.4, which are the respective optima for the microsomal- and cytosolic-like activities. BPO was separated from its diol by TLC of ether extracts of the incubation media as previously described (31). All fractions assayed were diluted so the content of Lubrol PX was 0.001% or lower, unless specified otherwise. Protein was measured using our previously described (31) modification of Bradford's method (32) with bovine serum albumin as standard.

Inhibition of hydrolysis. Inhibition of CSO hydrolase activity was performed by preincubation of 100 μl of purified epoxide hydrolase with 1 μl of varying concentrations of inhibitor in ethanol for 10 min at room temperature. Control samples received 1 μl of ethanol. Tubes were then transferred to a 37°C water bath and 1 μl of [^{3}H]CSO was added to initiate the reaction.

Diol formation was then determined as described above.

Antibody preparation. IgG against fraction HA A1 (anti-EH) was provided by Dr. James Hillman (Antibodies Incorporated, Davis, CA), and its production will be described in detail in a subsequent paper. In brief, female New Zealand White rabbits were injected (sc) with 100 μg of antigen in Freund's incomplete adjuvant on the back and legs. Blood was drawn for antisera after 3 weeks, and IgG fraction was prepared from chromatographic separation of serum over DEAE-Sephacel. Preimmune and specific IgG were found to be devoid of epoxide hydrolase activity for the substrates used in this study.

Immunoblot analysis. Proteins were separated on two (0.75 mm × 10 cm) 10% SDS-PAGE gels run concurrently on the same apparatus, essentially as described by Laemli (33). One gel was stained for protein with Coomassie brilliant blue. The other gel was used for overnight transfer to a nitrocellulose membrane, and processed as previously described (34). Anti-EH was used at a dilution of 1/100, and goat anti-rabbit IgG was conjugated to horseradish peroxidase at a dilution of 1/3000.

Immunoprecipitation. The immunoprecipitation of solubilized microsomes was carried out with slight modifications to our previously described method (35). Forty microliters of microsomes (0.3 mg protein/ml) was mixed with 100 μl of rabbit IgG (1.54 mg/ml). The latter was a mixture of 0–100 μl of anti-EH and 100–0 μl of preimmune rabbit IgG. This mixture was then incubated at room temperature for 3 h and then overnight at 4°C. One-half milliter of goat anti-rabbit IgG coupled to immunobeads was then added and allowed to incubate for 2 h and then centrifuged for 15 min at 1000*g*. For determination of percentage precipitated, aliquots of the supernatant were diluted with the appropriate buffer and assayed as described above. As a test for inhibition of activity by the antibody, the pellet in selected samples was resuspended with the remaining supernatant and aliquots were then taken for assay. In these samples the amount of activity removed prior to resuspension was noted to determine expected recovery. For the immunoprecipitation of purified eluate HA A2, the above procedure was used except that the protein concentration was initially 0.12 mg/ml for epoxide hydrolase and 3 mg/ml IgG. The mixtures were made at one-half the volumes noted above.

RESULTS

Lubrol Solubilization of Microsomes

The hydrolysis of CSO and BPO to their respective diols was readily apparent in microsomes from two male rhesus monkey livers with relatively high specific activities of 60 and 92 nmol/min/mg protein, respectively. The activity for hydrolysis of TSO was also detectable, but at a considerably slower rate of 0.07 nmol/min/mg protein (Table I). These values are similar to those reported in a previous study on liver microsomes from a different Rhesus monkey. Solubilization of the microsomes with Lubrol PX resulted in a recovery of 85% of the protein. Subsequent assays were performed on microsomes or eluates which contained Lubrol PX. Dilution of samples so that Lubrol PX was at 0.002% and 0.001% consistently gave similar activities, while less dilute samples had lower activities, indicative of inhibition by Lubrol PX. This suggested that 0.001–0.002% Lubrol PX was below the minimal concentrations for this detergent to exert its inhibitory action on epoxide hydrolase. Unless specified otherwise, all assays were performed with Lubrol PX at a concentration of 0.001% or less. Under these conditions, similar recoveries (55–59%) for the hydrolysis of all three substrates were found (Table I). Some loss of activity occurred, as the percentages recovered in the pellets were 18, 2, and 7 for CSO, TSO, and BPO hydrolysis, respectively.

Purification of CSO Hydrolase

The microsomes solubilized in this fashion were used as the starting material for the purification of epoxide hydrolase by column chromatography as illustrated in Fig. 1. Epoxide hydrolase activity was monitored as the CSO hydrolase. A single peak of activity eluted from the initial column (DEAE-Sephacel) at 180–220 mM potassium phosphate, while two peaks eluted from the second column of CM-cellulose at 5 mM and 80–120 mM potassium phosphate, respectively. These were referred to as fractions CM A and CM B. Upon subsequent separation of these fractions over hydroxyapatite, fraction CM A separated into two peaks of activity, HA A1 (eluting at 5 mM) and HA A2 (eluting at 100–200 mM) while fraction CM B eluted as a single peak of activity, HA B, at 100–180 mM potassium phosphate (Fig. 2).

TABLE I

SOLUBILIZATION OF EPOXIDE HYDROLASE ACTIVITIES IN RHESUS MONKEY LIVER MICROSOMES WITH LUBROL PX

Substrate	Microsomes (nmol/min/mg)	Solubilized microsomes (nmol/min/mg)	Recovery (%)
CSO (pH 9.0)	60.0 ± 1.3	35.2 ± 5.3	58.7 ± 10.2
BPO (pH 9.0)	91.8 ± 9.9	50.2 ± 5.0	54.6 ± 11.8
TSO (pH 7.4)	0.069 ± 0.041	0.038 ± 0.031	55.1 ± 13.7

Note. Microsomes from livers of rhesus monkeys (at 3.0 mg protein/ml) were assayed for hydrolysis of *cis*-stilbene oxide (CSO); Benzo[a]pyrene-4,5-oxide (BPO), and *trans*-stilbene oxide (TSO) as described under Experimental Procedures before and after they were solubilized with 1% Lubrol PX. Values are the mean ± SD of triplicate assays from microsomes prepared from two different livers.

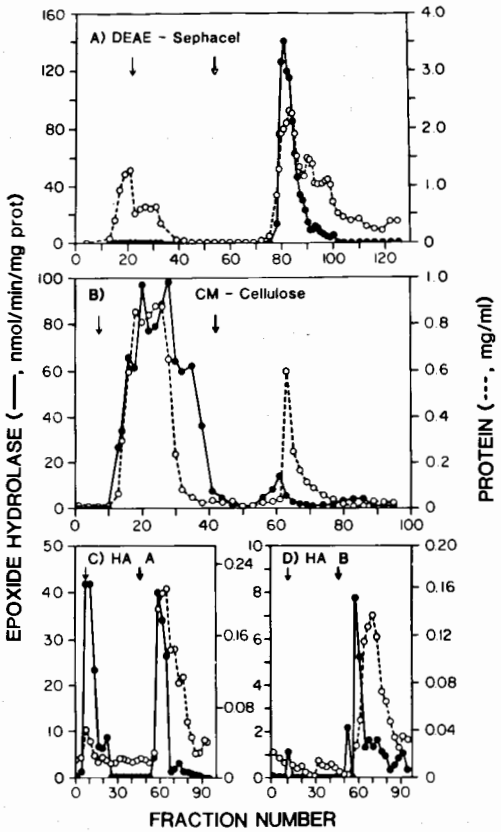

FIG. 2. Elution profiles during column chromatography of Rhesus monkey liver epoxide hydrolase. (A) Recovery on DEAE-Sephacel; (B) recovery of major peak from A over CM-cellulose; (C) recovery of the first peak from B after chromatography over hydroxyapatite; and (D) recovery of the second peak from B after chromatography over hydroxyapatite. Recovery of CSO hydrolase activity (—) and protein (---) are shown. Light arrows designate the start of wash buffer and heavy arrows the start of potassium phosphate gradient.

Comparative Recovery of CSO, BPO, and TSO Hydrolase

The recovery of protein and activity in these fractions is listed in Table II. The highest recovery of activity (6.6%), with CSO as substrate, and protein (3.2%) occurred in fraction HA H2, in which activity was enhanced 23-fold. A similar purification factor was found in fraction HA A1, but with only one-sixth the recovery. Fraction HA B contained only 0.2% of the initial activity and had only one-half the specific activity of the other two fractions (Table II). The recovery of BPO hydrolase was quite similar to that of CSO in all fractions measured. The hydrolysis of TSO, however, was selectively recovered in only a few of the fractions. The hydrolysis of TSO at pH 7.4 could be detected in both fractions eluting from the CM-cellulose column. After elution from the hydroxyapatite columns, hydrolysis of TSO was found to coelute with that of CSO in only fraction HA A2 (Table II). The total recovery of TSO hydrolase was actually less than that for the other two epoxides, but its enhancement in fraction HA A2 was greater, whether measured at pH 7.4 or 9.0 (Table II).

SDS-PAGE and Immunoblot Analysis

Polypeptides in the fractions recovered during purification were separated by SDS-PAGE (Fig. 3A). Of the three fractions recovered from the hydroxyapatite columns, HA A1 is the only one that appears homogeneous by this criterion. Fraction HA A2 has a few additional poly-

TABLE II

RELATIVE RECOVERY OF EPOXIDE HYDROLASE ACTIVITIES DURING PURIFICATION FROM RHESUS MONKEY LIVER MICROSOMES[a]

Protein		Epoxide hydrolase				
		CSO (pH 9.0)		% of CSO, pH 9.0 activity		
Fraction	% Recovered	Purification	% Recovered	BPO (pH 9.0)	TSO (pH 9.0)	TSO (pH 7.4)
Solubilized microsomes	100.0	1.0	100.0	142.6	0.016	0.118
DEAE	14.5	1.5	57.5	136.5	0.027	0.124
CM A	6.3	6.6	18.6	140.0	0.020	0.164
CM B	3.7	3.0	0.7	124.0	ND[b]	0.142
HA A1	0.6	17.9	1.0	130.0	ND	ND[c]
HA A2	3.2	22.9	6.6	140.0	0.078	0.344[c]
HA B	0.5	9.1	0.2	130.0	ND	ND[c]

[a] Fractions were recovered from peak column eluates as described in Fig. 1. Control values for activities are given in Table I.

[b] ND = not detectable.

[c] The sensitivity of the assay for TSO hydrolysis at pH 7.4 would have limited detectability of activity in HA A1, HA A2, and HA B to 0.13, 0.05, and 0.30% of CSO hydrolysis, respectively.

peptides, while fraction HA B has several. In all three fractions, however, the major polypeptide migrates at a similar rate (Fig. 3A). Comparison to molecular weight standards demonstrates that this polypeptide has an apparent molecular weight of 49,000. Fraction HA A1 was chosen as antigen for the production of antisera against purified rhesus monkey liver epoxide hydrolase.

The IgG fraction from this antisera was employed for immunoblotting of the companion gel to that described above. In solubilized microsomes and the fractions recovered from the hydroxyapatite column, a single colored band was found on the blots from each fraction (Fig. 3B). The bands also migrated with an apparent molecular weight of 49,000. The eluates from the CM-cellulose columns, however, did not have single bands. Eluate CM A had bands at approximately 25, 49, and 58 kDa, while eluate CM B had two bands, one at 49 and another at 63 kDa. As bands other than the 49-kDa band were neither apparent in solubilized microsomes, nor in the eluates from the hydroxyapatite column, they may well represent antigenically distinct impurities. In addition, the CM A and B eluates were tested against the antisera by immunoelectrophoresis and Ouchterlony double diffusion. During immunoelectrophoresis each eluate gave only a single arc, and during immunodiffusion the two eluates gave a single line of identity with the immunizing antigen, HA A1.[5] The three eluates from the hydroxyapatite columns, therefore, appear to have identical molecular weights, and immunochemical cross-reactivity.

Effect of Inhibitors, Lubrol PX, and Phospholipid

Additional tests concerning the identity of the eluates from the hydroxyapatite columns were performed to assess their enzymatic activities. The susceptibility of the three eluates of epoxide hydrolase to inhibition was tested using 1,1,1-trichloropropene oxide (TCPO) and 4-phenyl-chalcone oxide (4-PCO) with CSO as substrate (Table III). Protein was preincubated for 10 min with 5×10^{-7} to 5×10^{-5} M TCPO

[5] James Hillman, personal communication.

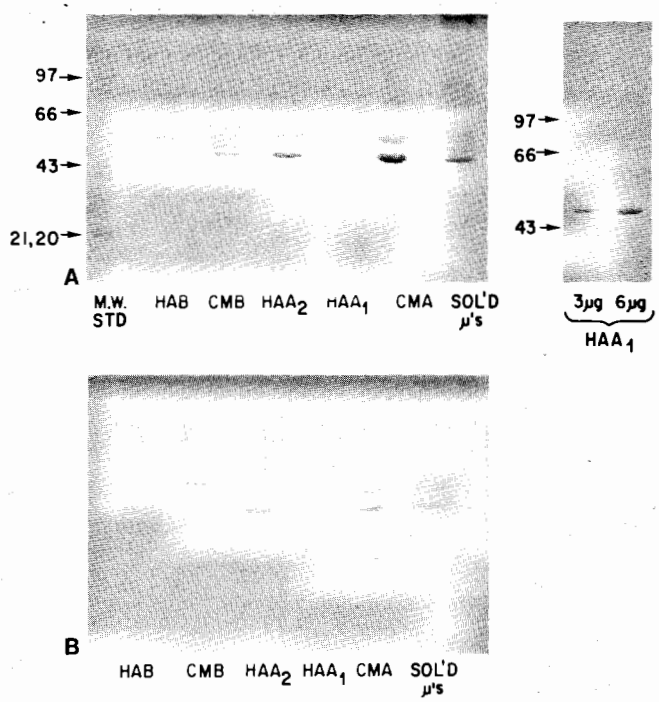

FIG. 3. Electrophorectic separation of solubilized microsomes and column fractions. (A) SDS–PAGE gel stained for protein with Coomassie brilliant blue; the two lanes (3 and 6 μg) of HA A1 (A, right) were run on a different gel, with the position of the standards indicated by arrows. (B) Immunoblot analysis of nitrocellulose membrane after transfer from gel run in tandem with that shown in A. Primary antibody was raised against eluate HA A1. The fractions separated and amount of protein used are as follows: Mic-solubilized Rhesus monkey liver microsomes (30 μg); CM A (15 μg); HA A1 (2 μg); HA A2 (5 μg); CM B (15 μg); and HA B (5 μg). These fractions are defined in more detail in Figs. 1 and 2, and in the text.

or 5×10^{-6} to 10^{-4} M 4-PCO prior to incubation with substrate. I_{50}'s calculated from these experiments demonstrated that HA A1 and HA B had a similar susceptibility to TCPO while the I_{50} for HA A2 was approximately a fivefold higher concentration. All three fractions were less susceptible to 4-PCO, but in this case, HA B was the most, HA A2 intermediate, and HA A1 not susceptible to inhibition by this compound (Table III).

In addition to the effects of inhibitors on activity, the effect of environment on the activity was tested by measuring CSO and TSO hydrolase activity in the presence of phospholipid and Lubrol PX (Table IV). The effect of these additives was dependent upon the eluate and substrate tested. The hydrolysis of CSO was reduced to only 67% of controls in fraction HA A1 but to 5% of controls in fractions HA A2 and HA B in the presence of 0.01% Lubrol PX. Addition of egg yolk phosphatidylcholine at 0.2 mg/

TABLE III

DIFFERENTIAL INHIBITION OF CSO HYDROLYSIS IN PURIFIED FRACTIONS OF MICROSOMAL EPOXIDE HYDROLASE[a]

Fraction	I_{50} (M) for CSO hydrolysis	
	TCPO	4-PCO
HA A1	1.9×10^{-5}	NI[b]
HA A2	1.1×10^{-4}	2.1×10^{-3}
HA B	1.7×10^{-5}	1.3×10^{-4}

[a] I_{50}'s were determined from means of four experiments as described under Experimental Procedures.
[b] NI = no inhibition detected.

mg protein significantly elevated the activity in fractions HA A1 and HA B, while activity in fraction HA A2 was unaffected. Hydrolysis of TSO was only detectable in eluate HA A2, and was reduced by the addition of Lubol PX and phospholipid (Table IV).

Immunoprecipitation of HA A2 and Microsomes

Eluate HA A2 was consistently different from the other two eluates in regard to enzymatic activity, in particular this fraction was capable of hydrolyzing TSO, which is generally regarded as specific for the cytosolic-like epoxide hydrolase. As this fraction was not homogeneous after SDS-PAGE, it is possible that the cytosolic-like epoxide hydrolase coeluted with the activity. In order to test for this possibility, HA A2 was immunoprecipitated with the monospecific anti-EH. At a ratio of IgG to protein that precipitated 100% of the CSO hydrolase activity in HA A2, all of the TSO hydrolase activity was also precipitated (Table V).

To test whether or not HA A2 may actually be the cytosolic-like epoxide hydrolase in rhesus monkey liver microsomes, immunoprecipitation was carried out using solubilized microsomes (Fig. 4). With increasing ratio of anti-EH IgG to microsomal protein, all of the activity toward CSO, and greater than 95% of the activity toward BPO was precipitated. In the same supernates, 40–50% of the activity toward TSO remained soluble. In the same experiments, the precipitated material was resuspended to allow assay of activity in the presence of antibody. Under these conditions, no significant inhibition of CSO hydrolase was found (Fig. 4). These experiments demonstrate that in rhesus monkey liver microsomes the hydrolysis of TSO can be separated into a fraction which is not and a fraction which is immunochemically similar to the microsomal-like epoxide hydrolase.

DISCUSSION

The results of this study demonstrate that rhesus monkey liver microsomal epoxide hydrolase activity, with both CSO and BPO as substrates, could be separated into distinct fractions by ion-exchange chromatography. The protocol used was similar to that employed by Guengerich *et al.* (26) to separate rat liver microsomal epoxide hydrolase. In that study and ours, activity eluted in the void volume and a high salt fraction from the CM-cellulose, with the void volume eluate being further resolved over hydroxyapatite. Lu *et al.* (23) also separated rat liver microsomal epoxide hydrolase over hydroxypatite, but only the high salt eluate was studied. More recently, Bulleid *et al.* (25) also separated rat liver microsomal epoxide hydrolase over CM-cellulose. In their study the void volume eluate was also resolved into two fractions

TABLE IV

EFFECT OF LUBROL PX AND PHOSPHOLIPID ON HYDROLYSIS OF CSO AND TSO IN PURIFIED FRACTIONS[a]

		Fraction (% control activity)		
Substrate	Addition	HA A1	HA A2	HA B
CSO	Lubrol	66.5 ± 28.9	4.4 ± 1.6	4.8 ± 6.7
	Phospholipid	214.0 ± 48.0	106.0 ± 10.0	174.0 ± 49.0
TSO	Lubrol	ND[b]	25.8 ± 13.5	ND
	Phospholipid	ND	48.9 ± 3.1	ND

[a] Lubrol PX was added to a final concentration of 0.01% and egg yolk phospholipid at 0.2 mg/mg protein. Values are the mean ± SD of two assays performed in triplicate at two different protein concentrations.

[b] ND = Not detectable.

TABLE V

IMMUNOPRECIPITATION OF HYDROLYTIC ACTIVITIES ACTING ON *cis*- AND *trans*-STILBENE OXIDE IN ELUATE HA A2[a]

	μg IgG/μg protein	CSO (pH 9.0) (nmol/min/ mg protein)	TSO (pH 7.4) (nmol/min/ mg protein)
Normal rabbit sera	60	49.8	0.716
anti-mEH	60	0.0	0.0

[a] Purified epoxide hydrolase was incubated with either normal rabbit or rabbit anti-EH IgG described under Experimental Procedures and activities for hydrolysis of CSO and TSO were determined on supernates.

over a second CM-cellulose column. Prior to our study, Guengerich *et al.* showed that primate liver epoxide hydrolase was resolved on ion-exchange columns using human tissue (26). While the resolution of epoxide hydrolase activity over ion-exchange columns has been repeatedly demonstrated, there has been a reluctance to accept this as evidence of multiple forms of the xenobiotic-metabolizing microsomal epoxide hydrolase.

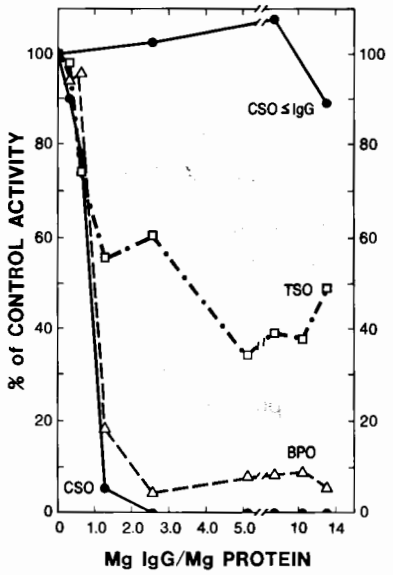

FIG. 4. Immunoprecipitation of epoxide hydrolase in solubilized Rhesus monkey liver microsomes. Lubrol PX-solubilized Rhesus monkey liver microsomes were incubated with rabbit anti-EH and preimmune IgG (total 12.5 mg IgG/mg protein), and then with goat anti-rabbit IgG immunobeads as described under Experimental Procedures. Supernates from immunoprecipitates were taken for the assay of CSO (●), TSO (□), and BPO (△) hydrolase activities. In addition pellets were also resuspended and CSO hydrolase activity measured in the presence of anti-EH IgG (●). The amount of anti-EH added is shown on the abscissa and activity recovered as a percentage of that found when preimmune IgG was used alone is shown on the ordinate.

Multiplicity of the microsomal epoxide hydrolase was initially inferred from differences in the inducibility and inhibition of different substrates. This difference was further demonstrated during purification of the enzyme as differential enrichment of activities was reported whether single of multiple peaks of activity were recovered (see references in (6) and (36)). Similar findings were found in this study on monkey liver epoxide hydrolase, particularly with the presence of TSO hydrolase activity restricted to eluate HA A2, and the differential inhibition of CSO hydrolase activity in the three eluates. It has been argued that these differences may arise from changes in the microenvironment of the enzyme, particularly from alterations in the enzyme to detergent or phospholipid ratios. In this study care was taken to assay hydrolase activities after dilution of the detergent Lubrol PX to concentrations which did not cause inhibition in this or previous studies. Furthermore, addition of Lubrol PX or phospholipid resulted in differential responses in the three eluates, unlike the findings of Bulleid *et al.* (25) with epoxide hydrolase purified from rat liver microsomes.

As found with previous studies using solubilized microsomes or purified enzymes

from rat and human liver (36–39), CSO and BPO hydrolase activities could be precipitated with antibody raised against a single eluate. These findings demonstrate that microsomal epoxide hydrolases must share antigenic sites, but do not rule out multiple isozymes. While monoclonal antibodies may offer a tool to unravel this question, none of those so far reported are capable of quantitative precipitation (40, 41). The demonstration of TSO hydrolase activity in only one of the fractions is a previously undescribed agrument for the structural uniqueness of the epoxide hydrolases separated by ion-exchange chromatography. Whether or not this structural difference arises from the presence of separate gene products or post-translational modification remains to be determined.

As the hydrolysis of TSO at pH 7.4 by fraction HA A2 occurs at a rate only 0.3% of CSO hydrolysis at pH 9.0, caution must be taken to eliminate the possibility that apparent TSO hydrolysis arose from either a minor component of HA A2, or from contamination of the [^{3}H]TSO with [^{3}H]CSO. First, it should be noted that the percentage of TSO/CSO hydrolysis, 0.3%, found in fraction HA A2 was much greater than the percentage 0.0012%, reported by Guenthner and Oesch (12) for purified mouse liver microsomal epoxide hydrolase. Second, the concomitant immunoprecipitation of TSO and CSO hydrolysis activity in HA A2 is inconsistent with a minor component of HA A2 being responsible for TSO hydrolysis. Third, if contamination of [^{3}H]TSO with [^{3}H]CSO resulted in apparent TSO hydrolysis, one would expect similar responses of both substrates to the effects of Lubrol PX and phospholipid, and a similar purification during column chromatography. Therefore, at least one fraction of rhesus monkey liver microsomal epoxide hydrolase appears to have discernible TSO hydrolase activity.

TSO hydrolase activity was completely precipitated in fraction HA A2 but not in solubilized microsomes. The latter finding demonstrates that a portion of this activity is due to an immunochemically distinct enzyme, which, based upon its activity toward TSO, may be similar to the cytosolic epoxide hydrolase. A similar immunologically distinct xenobiotic-metabolizing epoxide hydrolase has previously been described in mouse liver microsomes, and may also exist in human liver microsomes, based on substrate and inhibitor specificity data. The close immunochemical reactivity between human and monkey microsomal epoxide hydrolase (39) suggests that the antibody described in this study may also be useful to study the immunochemical relationship of human epoxide hydrolases.

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