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AFFINITY PURIFICATION OF CYTOSOLIC EPOXIDE HYDROLASE FROM HUMAN, RHESUS MONKEY, BABOON, RABBIT, RAT AND MOUSE LIVER

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Abstract—1. An affinity purification system based on elution of cytosolic epoxide hydrolase from a methoxycitronellyl thiol ligand with 4-azidochalcone oxide was applied to a variety of samples including liver from human, monkey, baboon, rabbit, rat and mouse as well as mammary gland from mouse.

2. Hepatic tissues yielded a major 58 kDa band on SDS–PAGE, but the system had to be modified slightly to remove a 33 kDa band for rat.

3. All of the affinity purified hydrolases showed similar properties with regard to substrate selectivity, pH dependence and mobilities on SDS-PAGE.

INTRODUCTION

Epoxide hydrolases (EC 3.3.2.3), catalyse the addition of water to endogenous and exogenous epoxides (Wixtrom and Hammock, 1985; Oesch, 1980; Lu and Miwa, 1980; Hammock *et al.*, 1980). In converting epoxides to 1,2-diols these enzymes greatly enhance the hydrophylicity of the substrate thus making further metabolism and/or rapid excretion possible. At present, four main forms of epoxide hydrolase are known and are characterized as cytosolic (cEH), microsomal (mEH), cholesterol (cholEH—also microsomal in location) and leukotriene epoxide hydrolase, distinguished in part by different molecular weight, substrate specificity, pH optima and immunoreactivities (Ota and Hammock, 1980; Guenther *et al.*, 1981; Watabe *et al.*, 1981; Levin *et al.*, 1983; McGee and Fitzpatrick, 1985). Although the microsomal form (mEH) has been purified and characterized from rat, mouse, rabbit and human liver (Wixtrom and Hammock, 1985), less is known of the cEH. CEH has been purified from rabbit (Waechter *et al.*, 1982), mouse (Gill, 1983; Meijer and DePierre, 1985) and human liver (Wang *et al.*, 1982), with a 0.5–10% yield and 180–550-fold purification. Classical techniques were used for these enzyme purifications which, in general, required large amounts of tissue, provided a low yield and were multistep. Recently, a one step purification method was developed utilizing a bioselective adsorbent for cEH offering a rapid, high yield, quantitative method for purification of cEH from mouse liver. The one step cEH purification technique requires only small amounts of tissue, yet gives a high yield of enzyme (Prestwich and Hammock, 1985). It was our intention to apply this method to human (*Homo sapiens*), rhesus monkey (*Macaca mulatta*), baboon (*Papio papio*), rabbit (*Oryctolagus cuniculus*), rat (*Rattus norvegicus*) and mouse (*Mus musculus*) liver in order to characterize and compare cEH activity among several species. We used substrates which have been shown to differentiate between cEH and mEH in Swiss–Webster mice (Wixtrom and Hammock, 1985) and initially tested them in unpurified microsomal and cytosolic fractions. Then, the same substrates were used with purified cEH and nonbinding fractions to establish substrate selectivity for all samples.

As a challenging application of the methodology, we selected mammary gland as an important and technically difficult extrahepatic site for cEH characterization, since EH activity has been observed in rodent mammary epithelium (Dent *et al.*, 1977; Greiner *et al.*, 1980). Known mammary carcinogens and mutagens may be substrates for mEH (Oesch, 1974; Lu *et al.*, 1979; Tay and Russo, 1981; Moore *et al.*, 1983) and epoxides of fatty acids are readily metabolized by cEH (Chacos *et al.*, 1983; Gill and Hammock, 1979), thus investigation of epoxide metabolism in the mammary gland may help to explain the influence of diet on occurrence of mammary preneoplasia and neoplasia in mice and rats (Abraham *et al.*, 1984).

EXPERIMENTAL PROCEDURES

Chemicals

trans-Stilbene oxide (TSO), *n*-dodecane (99%) and diethylmaleate (DEM) were purchased from Aldrich Chemical Co. (Milwaukee, WI). cis-Stilbene oxide (CSO) was made in-house from cis-stilbene (purchased from Aldrich). 4-Azidochalcone oxide was prepared as described (Prestwich and Hammock, 1985). Citronellol epoxide was prepared by oxidation of citronellyl acetate with *m*-chloroperbenzoic acid in chloroform followed by ester hydrolysis. Stock solutions of inhibitors were prepared at 0.05 M in ethanol. Bovine serum albumin (BSA-fraction V) was obtained from Sigma Chemical Co. (St Louis, MO). Bio-Rad protein dye reagent was purchased from Bio-Rad laboratories (Richmond, CA). ACS Scintillation cocktail was purchased from Amersham (Arlington Heights, IL).

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Radiochemicals

(^{3}H)CSO and TSO were prepared as described (Gill *et al.*, 1983) by base treatment of the corresponding chlorohydrin obtained by desylchloride reduction using sodium borotritide. (^{3}H)Benzo(a)pyrene-4,5-oxide (BPO) (288 mCi/mmol) and unlabeled BPO were gifts.

Column preparation. Sepharose CL-6B (Pharmacia, NJ) was activated with 1,4-butanediol diglycidyl ether (Aldrich) according to literature procedures (Porath, 1974; Scouten, 1984). 7-Methoxycitronellyl thiol (MCT) was prepared in four steps (Prestwich *et al.*, 1984) and added to the epoxy-activated resin as described (Prestwich and Hammock, 1985). The column, normally stored at 4°C in absolute ethanol containing butylated hydroxy anisole (BHA; 0.2% w/v), was washed with 10 vol. of buffer prior to its use for enzyme purification.

Tissue samples

Human samples A and B were obtained by Dr Boris Ruebner, M.D., University of California, Davis Medical Center, Sacramento, CA. Histologically normal sections of liver were taken from patient A, a 51-year-old woman with cancer of the bile duct and patient B, a 53-year-old man with metastatic cancer of the colon.

Other primate samples were obtained through the Northern California Primate Research Center, Davis, CA. Liver samples were removed from a normal 6-year-old male Rhesus monkey who died accidentally of a broken neck; a 15-year-old female Rhesus monkey who died of a stroke and an 18-year-old female baboon who died of old age.

Male Sprague-Dawley rats (270–310 g) were purchased from Charles River. The 2.5 kg female New Zealand white rabbit was received from Herbert's (Plymouth, CA). Pregnant female Balb/c mice were received from the laboratories of Dr L. J. Faulkin, University of California.

Rats, rabbit and mice were housed in steel cages in environmentally controlled rooms (23 + 1.5°C; 14:10 hr light:dark). Food (for the rabbit and mice: Purina laboratory chow) and water were provided *ad libitum*. Food for the clofibrate induced (CF rat) and control rats was ground Purina laboratory chow. Clofibrate was dissolved in corn oil and mixed into ground chow at a concentration of 0.5% (w/w). Control animals received ground chow with similar amounts of corn oil added (5 ml/100 g). Experimental animals were given clofibrate-containing diet for 14 days.

Enzyme preparation

Human samples were frozen at −80°C until homogenized while other primate samples were received on ice and worked up within 30 min. Rat and rabbit livers were removed after the animals were terminated by exsanguination. Mice were terminated by cervical dislocation then liver and all mammary tissue were removed.

Livers were perfused with 1.15% KCl through hepatic portal vein or a major vessel in the case of primate samples and homogenized in sodium phosphate buffer (76 mM, pH 7.4) or 0.25 M sucrose–10 mM Tris–HCl pH 7.4 (primate samples) plus 3 mM 2-mercaptoethanol. Rat livers were pooled from groups of animals, mouse livers and mammary glands from mid-to-late pregnant Balb/c mice were pooled from 15 animals; and a single rabbit was sampled. Samples were weighed and minced, then homogenized in phosphate or Tris buffer with a Polytron for 20 sec. Samples were centrifuged at 105,000 *g* for 60 min. The cytosol (105,000 *g* supernatant) was collected after drawing off the fatty layer. Microsomal pellets were resuspended in 50 mM Tris–HCl (pH 9), 129 mM KCl and recentrifuged. Washed microsomes were resuspended in phosphate buffer (76 mM, pH 7.4) or 0.25 M sucrose–10 mM Tris–HCl plus 3 mM 2-mercaptoethanol (pH 7.4), to give 25–50% homogenate based on the initial homogenization w/v. Appropriate dilutions of cytosolic and microsomal fractions were made to yield linear hydration of substrate with time.

Enzyme assays

Unpurified cEH, column fractions, and mEH activities were determined by partition assay with TSO and CSO as previously described [(^{3}H)TSO and CSO 0.05 mM; 68 mCi/mmol] (Wixtrom and Hammock, 1985). The activity of cEH was determined with TSO in 76 mM sodium phosphate buffer (pH 7.4), and mEH with CSO and BPO in 100 mM Tris–HCl (pH 9). The hydrolysis of BPO was determined by the thin layer procedure of Jerina *et al.* (1977). (^{3}H)BPO was diluted with unlabeled substrate to give a final substrate concentration of 0.05 mM. Substrates used in these assays are known to be soluble at the concentrations indicated (Hammock and Hasagawa, 1983; Wixtrom and Hammock, 1985) and their low rates of chemical hydration have been previously described (Long and Pritchard, 1956; Pritchard and Long, 1956). All reported values were corrected for nonenzymatic reaction and were obtained under conditions of maximal velocity. Protein concentrations in the various fractions were determined using a modification of the Bradford method (Bradford, 1975).

All samples were screened for interfering glutathione levels since the presence of gluthathione (GSH) in the cytosolic fractions can lead to artifactually high levels of radioactivity in the aqueous fraction of the partition assay (Mullin and Hammock, 1980). Therefore, all samples were assayed with and without DEM (a depleter of GSH) to control for this problem. Only the rat was found to contain interfering levels of GSH based on discrepancies between rates found with and without DEM. Thus, all nonbinding, washed and unpurified cytosolic fractions from rat were incubated for 10 min at 37°C in 0.5 mM DEM prior to cEH enzyme assays. Selected samples were dialysed 12 hr before assay and the results found comparable with DEM depletion. DEM at this concentration was not found to influence the activity of the cEH (Moody *et al.*, 1986).

Polyacrylamide gel electrophoresis

Molecular weight determinations and homogeneity of cEH eluted with 4-AZCHO from each sample was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). A Hoefer SE600 Vertical Slab Gel unit was employed to conduct discontinuous SDS–PAGE according to Laemmli (1970) on 0.75 mm gels. Approximately 2–6 μg of protein per lane were added for tracks shown in Fig. 1, however, all samples were, at one time or another, run in concentrations ranging from 1 to 20 μg per lane. Following electrophoresis at 4°C at 15 mA/gel, gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 1:1 methanol:water containing 10% v/v acetic acid, then destained with ethanol:14% aqueous acetic acid (1:2).

Affinity chromatography on MCT-Sepharose

Affinity purifications were performed at 4°C. Routine separations were done using a 300 μl bed of MCT-Sepharose in a 1 ml tuberculin syringe equilibrated with a 76 mM sodium phosphate buffer, pH 7.4 containing 0.1 mM sodium EDTA and 0.1 mM dithiothreitol (DTT). The column where male rhesus cEH was purified was a larger version of approximately 5 ml of MCT column gel in a 1 × 8 cm column.

Cytosol prepared from each tissue sample was diluted to 4% (w/v) in the above buffer for application to the column. Phenylmethylsulphonylfluoride (0.1 mM PMSF) added to sodium phosphate buffer, used throughout purification, eliminated minor bands seen on SDS–PAGE. Diluted cytosol was pumped onto the MCT column at 12–14 ml/hr using a peristaltic pump, then recirculated in a closed system continuously over the column for 18 hr. This recirculating or nonbinding fraction was collected and saved. The column was then washed with 12–20 ml of the above mentioned buffer. Buffer was held in a reservoir to

wash through the column at 12–14 ml/hr then collected and saved. Elution of cEH was accomplished using 3–5 ml of the inhibitor 4-AZCHO at 0.5 mM. Inhibitor in buffer containing 0.01% ethanol was prepared from ethanol stock solution immediately prior to use, since at this high concentration in aqueous solution the 4-AZCHO does not completely dissolve. The 3–5 ml inhibitor was was followed by a 2 ml buffer chase lacking the inhibitor. This fraction, the eluting fraction plus chase were collected and saved. All fractions: nonbinding fraction, buffer wash and eluting fraction were collected on ice from the column and kept on ice until they were assayed.

Dialysis

Dialysis was done using Spectrapor 2 membrane tubing (mol. wt cutoff 12,000–14,000). For measurement of cEH activity, the enzyme was stabilized by addition of 76 mm sodium phosphate buffer (pH 7.4) containing 0.1 mM ethylenedinitro-tetraacetic acid (EDTA) and 0.1 mM DTT. Aliquots of each eluting fraction were dialysed with and without 1% BSA for approximately 18 hr at 4°C.

Estimation of purity of cEH on gels. SDS-gels containing the chromatographed elution fraction from each sample were scanned on an LKB model 2202 Ultrascan laser densitometer. Electrophoretic purity of cEH was based on peak areas printed out by a Hewlett–Packard 3390A Integrator for each band appearing on the gels.

RESULTS

Cytosolic and microsomal epoxide hydrolase activity in subcellular fractions

Microsomes and cytosol were tested with TSO and CSO, pH 7.4 and with CSO and BPO pH 9.0 (Table 1) to see if these substrates and incubation conditions were of diagnostic value in distinguishing among the different epoxide hydrolase activities in subcellular fraction and to compare values with those of previous studies. In male Swiss–Webster mice, substrates and pH conditions have been established as diagnostic for mEH (CSO pH 9.0 and BPO pH 9.0) and cEH (TSO pH 7.4) activity (Oesch, 1980; Lu *et al.*, 1979; Lu and Miwa, 1980; Hammock and Hasagawa, 1983; Wixtrom and Hammock, 1985). All reported values were corrected for nonenzymatic hydration and were obtained under conditions of maximal velocity.

In most cases, BPO and CSO at pH 9.0 represented the substrate and pH conditions which promoted the highest mEH specific activity. It has been demonstrated that epoxides derived from polycyclic aromatic hydrocarbons make excellent substrates for mEH (Oesch, 1980; Lu and Miwa, 1980; Wixtrom and Hammock, 1985). CSO at pH 9.0 was also a good substrate for mEH activity, the values, in many cases, were comparable to those obtained with BPO pH 9.0. These data support an inter-species mEH selectivity for BPO and CSO at pH 9.0. In each case, CSO activity in microsomes was less at pH 7.4 than at pH 9.0 indicating the pH 9.0 enzyme was more important than cEH-like activity in the microsomes (Kawabata *et al.*, 1983). However, the ratios varied, indicating involvement of multiple enzymes or different pH profiles. There was low hydrolytic activity on TSO pH 7.4 in microsomes for all species relative to that observed for CSO and BPO as expected.

Data for cytosolic fractions showed BPO hydrolysis in most cases was similar to or greater than hydrolysis of CSO pH 9.0. With CSO pH 7.4 the hydrolysis rate was, for the majority of the samples, lower than that of CSO pH 9.0. CF rat, rabbit, Balb/c liver and mammary gland showed highest cytosolic activity with TSO and the expected sub-

Table 1. Comparisons of microsomal and cytosolic epoxide hydrolase activities* among samples using different substrates and pH conditions

		Microsomes				Cytosol			
Sample	Sex	BPO† pH 9.0	CSO† pH 9.0	CSO‡ pH 7.4	TSO‡ pH 7.4	BPO† pH 9.0	CSO† pH 9.0	CSO‡ pH 7.4	TSO‡ pH 7.4
Rhesus	Male	101 (1220)§	72 (863)	26 (706)	2.5 (30)	3.4 (247)	1.3 (93)	1.3 (83)	0.95 (67)
Rhesus	Female	28 (387)	29 (405)	9.0 (126)	0.22 (3.1)	3.0 (132)	1.5 (66)	0.89 (41)	0.54 (22)
Baboon	Female	42 (629)	37 (556)	30 (445)	0.57 (8.6)	0.75 (42)	1.8 (101)	1.4 (78)	1.2 (68)
Human A	Female	56 (620)	64 (704)	53 (581)	0.87 (9.6)	0.09 (5.0)	3.6 (198)	1.8 (99)	2.4 (132)
Human B	Male	40 (402)	43 (432)	41 (407)	0.95 (9.5)	0.10 (4.9)	2.8 (126)	1.3 (55)	0.63 (28)
CF Rat	Male	40 ± 2.8 (216)	36 ± 3.8 (174)	–‖	0.50 ± 0.010 (2.1)	0.34 ± 0.027 (34)	0.10 ± 0.002 (10.2)	–‖	0.60 ± 0.089 (20.4)
Control rat	Male	32 ± 1.5 (150)	24 ± 2.0 (150)	–‖	0.40 ± 0.029 (2.0)	0.40 ± 0.017 (23)	0.20 ± 0.005 (8.6)	–‖	0.10 ± 0.009 (5.0)
Rabbit	Female	8.4 (201)	18 (423)	14 342	0.10 (2.4)	1.3 (136)	1.3 (134)	0.89 (94)	2.6 (273)
Balb/c liver	Female	19 (263)	2.2 (30)	1.5 (20)	0.27 (3.6)	0.08 (6.8)	0.11 (9.4)	0.31 (26)	2.0 (173)
Balb/c mammary gland (mid pregnant)	Female	0.19 (0.93)	0.13 (0.89)	0.12 (0.84)	0.08 (0.56)	0.02 (0.66)	0.02 (0.66)	0.02 (0.66)	0.13 (4.3)

*Enzyme activity is expressed as nmol/min/mg protein. All rates were determined from the linear region of a time course and within assay variation was <6% of the values reported. Values reported are means of experiments performed in triplicate from (1) a single liver in the case of human, nonhuman primate and rabbit; (2) six livers each were pooled for clofibrate induced and control rats; (3) 15 livers were pooled for Balb/c mice and from the same animals' mammary tissue was also pooled; (4) six control and clofibrate treated rat livers were worked up individually and values are reported with standard deviation.

†Assays run with 0.1 M Tris–HCL, pH 9.0 buffer.

‡Assays run with 76 mM sodium phosphate, pH 9.0 buffer.

§Activity expressed as nmol/min/g tissue for the purpose of comparison among samples.

‖Assay not run in this species.

Table 2. Distribution of protein and enzyme activities in nonbinding and eluting fractions from the MCT column using *trans*-stilbene oxide (pH 7.4) as substrate: a comparison among samples

		Starting cytosol		% of total recovery in nonbinding fraction		% of total recovery in eluting fraction	
Sample	Sex	Total protein (mg)	Total activity (nmol/min)	protein	Enzyme activity	Protein	Enzyme activity
Rhesus	Male	1335	565	92	56	0.16	48
Rhesus	Female	77	21	99.7	18	0.23	20
Baboon	Female	157	109	99	16	0.43	58
Human A	Female	80	88	91	54	0.22	25
Human B	Male	108	23	66	49	0.13	49
CF Rat	Male	134	90	94	16	0.37	47
Control rat	Male	145	23	99	25	0.17	29
Rabbit	Female	153	177	87	32	0.22	18
Balb/c (Liver)	Female	65	250	61	37	0.28	5.0*
Balb/c (Mammary gland)	Female	94	8.6	99	14	0.11	56

*Subsequent studies have shown that the recirculation procedure yields low recovery of cEH for mouse liver compared to the method described by Prestwich and Hammock (1985), but it is reported here to facilitate comarison with other species.

strate preference. The substrate selectivity of the other samples were not as sharply differentiated.

Cytosolic epoxide hydrolase purification

When cEH was purified, the percent recovery differed among samples, but since column conditions originally were optimized for cEH from Swiss–Webster mouse, some variation was expected for different species (Table 2). Variation also was expected based on other studies which demonstrated the optimum amount of MCT gel varied with the specific and total enzyme activity applied (Prestwich and Hammock, 1985). However, a decision was made to use a constant gel bed volume for all samples from a single MCT preparation. Some variations was also expected based on species, strain and sample differences and the fact that all colums were not run simultaneously.

Some activity with TSO pH 7.4 eluted with the nonbinding fraction (i.e. did not stick to the column) along with the majority of protein (Table 2). However, the eluting fraction which contained from 0.1 to 0.43% of total protein was highly active with TSO pH 7.4 and thus, provided evidence that in this

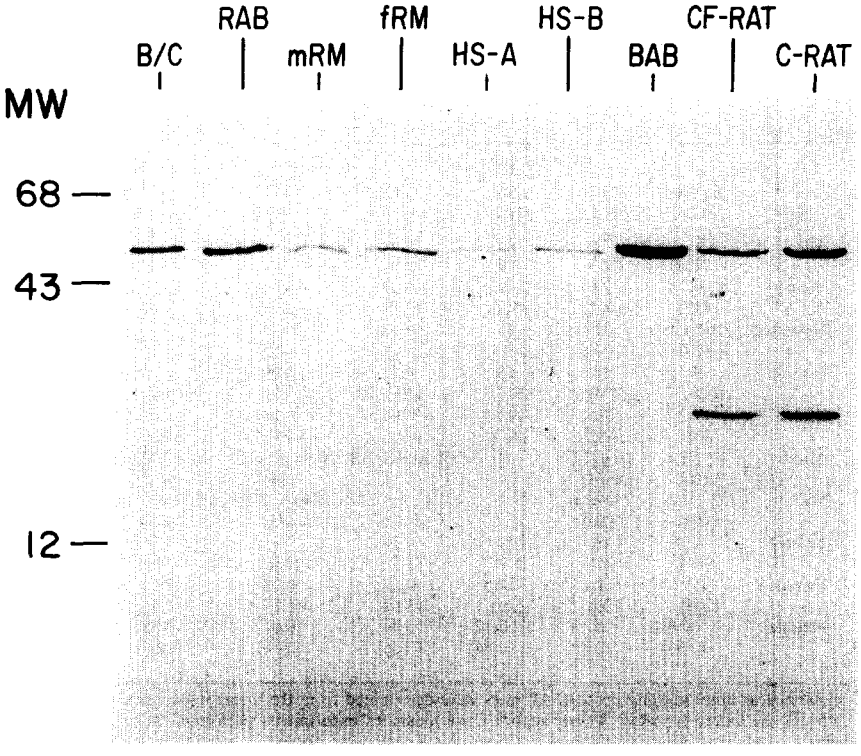

Fig. 1. SDS–PAGE of affinity purified cytosolic epoxide hydrolase from several species. In each case the band at 58–60 kDa represents the fraction eluted from the MCT column by 4-azidochalcone oxide. Abbreviations include: Balb/c liver (B/C), rabbit (RAB), male rhesus monkey (mRM), female rhesus monkey (fRM), human sample A (HS-A), human sample B (HS-B), baboon (BAB), clofibrate induced rat (CF-rat), control (C-rat).

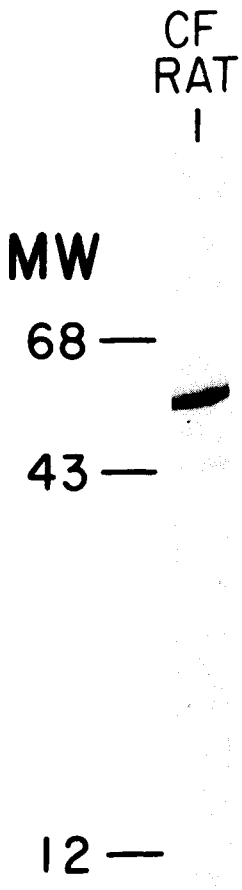

Fig. 2. SDS–PAGE of affinity purified cytosolic epoxide hydrolase from the clofibrate induced rat. In this case the band at 58–60kDa represents the fraction eluted from the MCT column by citronellol epoxide.

fraction the cEH was enriched greatly. In addition, in Table 2 it can be observed that the same protein is responsible for TSO hydrolysis in both the control rat and the clofibrate treated rat. In rat, TSO hydrolysing proteins appear to be induced by clofibrate as we have observed in mouse (Hammock *et al.*, 1986).

Homogeniety and molecular weight of eluted cEH was assessed by polyacrylamide gel electrophoresis in the presence of SDS. Figure 1 illustrates the apparent purity of cEH eluted with 4-AZCHO. All samples showed a major band at 58 kDa for cEH which under those conditions agreed with values of 57–59 kDa for rabbit, human and mouse liver cEHs (Waechter *et al.*, 1982; Wang *et al.*, 1982; Gill, 1983; Meijer and Hammock, 1985; Prestwich and Hammock, 1985). Others have shown by gel filtration a molecular weight of approximately 130 kDa for native cEH (Gill and Hammock, 1980; Meijer *et al.*, 1982), so it was proposed (Wang *et al.*, 1982) that cEH consists of two identical monomeric units. With the primate, human, rabbit and mouse liver samples one strong band was observed. CF and control rats showed a secondary major band at 33.4 kDa which appeared to be as dark as the 58 kDa band even when PmSF was used in buffers during cEH purification. A similar pattern on SDS-PAGE with a 33.4 kDa band was obtained from the mammary gland preparation. The major band appeared to have the same Rf as the other cEH samples. We were able to approximate purity of major bands by using an LKB Laser Densitometer (Table 3). All samples showed a purity of 83–98% by band density, except CF and control rat and mammary tissue which had an additional band at 33.4 kDa.

Table 3. Determination of % protein at 58 kDa by Laser-Densitometer

Sample	Sex	Fraction % at 58 kDa	Fraction % at 33.4 kDa
Rhesus	Male	85 ± 2‡	–
Rhesus	Female	92 ± 3‡	–
Baboon	Female	83 ± 5†	–
Human A	Female	87 ± 2§	–
Human B	Male	94 ± 5‡	–
CF Rat	Male	53 ± 7‡	35 ± 11‡
Control rat	Male	49 ± 0‡	51 ± 4‡
Rabbit	Female	89 ± 3‡	–
BALB/C liver	Female	98 ± 2‡	–

*The entire gel track was scanned using an LKB Laser Densitometer and peak area integrated on a Hewlet–Packard 3390A. The fraction of 58 kDa was estimated based on total peak area with no correction for gel background.
†Average of two gels.
‡Average of three gels.
§Average of four gels.

The lower band was not detected in purified rat cytosol when 0.5 mM citronellol epoxide was used as the eluting inhibitor (Fig. 2). It is interesting that this compound was less effective than 4-AZCHO as eluting inhibitor when used with mouse cytosol. This observation demonstrated that conditions can be varied slightly to obtain pure enzyme from sources with very low specific activity.

Comparison of CSO and BPO pH 9.0 activities in column fractions among species

Both CSO and BPO pH 9.0 showed a high percentage recovery of enzyme activity in the nonbinding fractions (including buffer wash) (Table 4) for all samples except mammary gland. CSO (mEH-like) activity recoveries ranged from 59% in mammary gland to 99% in human sample B and BPO (mEH-like) activity recoveries ranged from 87% in female rhesus to 100% in several samples (nonbinding fractions and eluting fractions from Balb/c liver and mammary gland were not tested with BPO pH 9.0). Eluting fractions were inactive with BPO in all cases and also showed low activity with CSO pH 9.0, accounting for only 0.0–3.5% of total starting activity. CSO pH 9.0 was tested on mammary gland fractions, but the activity was not detectable over background.

DISCUSSION

A major goal of this study was to test the ability of the MCT column to purify the cEH from a variety of species and to determine if proteins with the same apparent molecular weight were responsible for TSO hydrolysis. Initially, however, we chose to demonstrate the crude cytosolic protein added to the MCT column gave enzyme activities similar to literature values for all samples previously studied. Additionally, it was necessary to show that by cautious use

Table 4. Distribution of protein and enzyme activities in nonbinding and eluting fractions from the MCT column using *cis*-stilbene oxide and benzopyrene-4,5-oxide, pH 9.0 as substrate: a comparison among samples

		CSO pH 9.0 (nmol/min)	BPO pH 9.0 (nmol/min)	CSO pH 9.0 (nmol/min)		BPO pH 9.0 (nmol/min)			
Sample	Sex	Total activity in starting cytosol*	% Total activity in nonbinding fraction†	Total activity in starting cytosol*	% Total activity in nonbinding fraction†	Total activity in eluent fraction‡	% Total activity in eluent fraction§	Total activity in eluent fraction‡	% Total activity in eluent fraction§
Rhesus	Male	1861	98	523	100	10	0.56	ND‖	0
Rhesus	Female	135	89	230	87	1.4	1.0	ND	0
Baboon	Female	217	88	476	100	7.6	3.5	ND	0
Human A	Female	276	91	286	92	2.3	0.83	ND	0
Human B	Male	157	99	92	100	1.2	0.78	ND	0
CF Rat	Male	179	98	133	100	5.0	2.8	ND	0
Control rat	Male	50	98	53	100	0.94	1.9	ND	0
Rabbit	Female	277	88	219	100	1.5	0.54	ND	0
Balb/c (Liver)	Female	46	59	–¶	–	1.3	2.9	–	–
Balb/c (Mammary gland)	Female	ND	ND	–	–	ND	ND	–	–

*Total units in cytosol added to column.
†% units in nonbinding fraction (protein which did not stick to the column).
‡Total units in cEH eluting fraction.
§% units in cEH eluting fraction.
‖ND = not detectable.
¶BPO pH 9.0 not run for Balb/c liver and mammary gland.

of substrates and pH conditions we could distinguish between two major types of epoxide hydrolase (cEH and mEH). The ability to clearly distinguish between cEH and mEH activity becomes obvious when substrate selectivity and pH variations were employed as demonstrated with MCT column fractions for partial evidence of enzyme identity. Literature values for mEH and cEH activities from human and nonhuman primates showed a broad range of activities, which our data also confirmed. In general, our data agreed closely with previously reported values (Gill *et al.*, 1983). Mertes *et al.* (1985), found mEH rates with BPO pH 9.0 of 9.81 nmol/min per mg from patients with histologically normal liver, not treated with drugs 20 days prior to biopsy, but with cholelithiasis. Histologically normal human livers in our study had been removed from drug treated patients with bile duct carcinoma or metastasized colon carcinoma. BPO hydrolysis rates with mEH from these patients were 40–56 nmol/min/mg. On the other hand, Mertes *et al.* (1985) could not detect mEH activity in microsomes with TSO pH 7.4 whereas we showed low, but measurable hydrolysis (0.87–0.95 nmol/min per mg) in washed microsomes. Our data also can be contrasted to that of Wang *et al.* (1982) who purified cEH and mEH from liver of a human who died of intracranial hemorrhange. In this case the unpurified cEH activity with TSO pH 7.4 was 2.8–10 times greater than hydrolysis rates for unpurified cEH in this study and mEH activity with TSO pH 7.4 was 50 times higher than we observed. Their mEH activity with TSO was 7.6 times greater than their cEH activity with TSO. It may be difficult to establish concrete patterns for human mEH and cEH since sex, age, race, health status, drug regimen, tissue preservation methodology and other factors affect the final enzyme activities.

As indicated before (Wixtrom and Hammock, 1985) CSO and BPO appear to be hydrolysed at similar rates at pH 9.0 in microsomes of various species (Table 1). The ease of the CSO assay thus may make it the preferable substrate. However, the data obtained from rabbit and Balb/c mice indicate that a different substrate selectivity or different ratio of contributing enzymes could be involved.

In comparison of hydrolysis of CSO at pH 9.0 versus CSO at pH 7.4, the hydrolysis of CSO was, in general, higher in the cytosol at pH 9.0 that at pH 7.4. This observation may indicate that a microsomal-like enzyme was responsible for most of the hydrolysis of CSO. The relative cytosolic activity with BPO and CSO varied among the species examined with the discrepancy being most marked in the case of the human samples. Many studies, including some from this laboratory (Kapitulnik *et al.*, 1977) have shown there is a clear although sometimes small pH dependence for the microsomal hydrolase.

Cytosolic fractions, characterized as described above, were passed through the MCT gel and the majority of the TSO activity bound on the column while the fraction active in CSO and BPO hydrolysis washed through. Columns were eluted with 4-AZCHO, which proved to be selective for eluting an enzyme with high TSO (cEH) activity for all species and samples indicating similarity among the enzymes. Cytosolic EH appears to be one of a class of enzymes increased by peroxysome proliferating agents such as clofibrate. It appears that the same enzymes are responsible for TSO hydrolysis in the clofibrate induced and uninduced rat. This indicates that true induction occurs with clofibrate treatment as we have observed in mouse (Hammock *et al.*, 1986). The percent total activity of cEH from mammary gland was significantly lower than that of liver with all substrates. This was not unexpected since unpurified cEH from extrahepatic tissue has often been shown to be less active than unpurified cEH of liver (Loury *et al.*, 1985). High TSO activity was always found in the eluting fraction containing the purified enzyme, but a significant amount of TSO activity remained in nonbinding fraction, indicating that columns were overloaded.

By use of the MCT–Sepharose column, we have been able to clarify some of the confusing obser-

vations such as "mEH-like" activity in the cytosol. The 4-AZCHO eluting fractions showed no BPO and little CSO activity where significant activities for these substrates was observed in the nonbinding fraction as well as in the starting cytosols. Therefore, once cEH was purified and contaminating "mEH-like" activity was removed, high selectivity for TSO pH 7.4 by cEH was observed.

In all cases, by visualization and by LKB Densitometer impurities were minor. Only in rat and mammary gland was there more than one major band evident on SDS–PAGE. PMSF added to unpurified cytosol and all buffer solutions during cEH purification helped to eliminate minor bands on SDS–PAGE. However, even with use of PMSF, rat showed two main bands when cEH was eluted with 4-AZCHO. By varying column conditions and using citronellol epoxide for elution, apparently homogeneous cEH was obtained from control and clofibrate-fed rats using the one-step MCT column. The protein patterns observed by SDS–PAGE of the 4-AZCHO eluting fraction and the similar enzyme activity patterns for each sample lend strong evidence that they are the same enzyme.

The information presented in this work can serve as an example of how useful the one step MCT method is for purifying cEH from many species with relatively small tissue samples or from extrahepatic samples where cEH is very low. At present, it would be difficult to choose a species to serve as a model for human since a great deal of variation in enzyme activity exists among species. However, the cEH isolated from all species appears to be very similar in behavior on the column, molecular weight, and with regard to hydrolysis of the three substrates tested.

Of great importance to human cancer research is defining and understanding the nature and character of enzyme systems which metabolize chemicals. The data in this report on the hydrolysis of three potentially diagnostic substrates may aid in selecting useful experimental models for metabolism studies, and the MCT procedure now appears generally applicable for the purification of an enzyme important in epoxide hydrolysis.

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