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Comparison of Crude and Affinity Purified Cytosolic Epoxide Hydrolases from Hepatic Tissue of Control and Clofibrate-Fed Mice[1]

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An affinity purification procedure was developed for the cytosolic epoxide hydrolase based upon the selective binding of the enzyme to immobilized methoxycitronellyl thiol. Several elution systems were examined, but the most successful system employed selective elution with a chalcone oxide. This affinity system allowed the purification of the cytosolic epoxide hydrolase activity from livers of both control and clofibrate-fed mice. A variety of biochemical techniques including pH dependence, substrate preference, kinetics, inhibition, amino acid analysis, peptide mapping, Western blotting, analytical isoelectric focusing, and gel permeation chromatography failed to distinguish between the enzymes purified from control and clofibrate-fed animals. The quantitative removal of the cytosolic epoxide hydrolase acting on *trans*-stilbene oxide from 100,000*g* supernatants, allowed analysis of remaining activities acting differentially on *cis*-stilbene oxide and benzo[*a*]pyrene 4,5-oxide. Such analysis indicated the existence of a novel epoxide hydrolase activity in the cytosol of mouse liver preparations. © 1986 Academic Press, Inc.

Compounds containing the oxirane functionality (i.e., epoxides) include important biosynthetic intermediates such as squalene oxide and leukotriene A_4, environmental contaminants such as heptachlor epoxide, industrial intermediates like *bis*-phenol A diglycidyl ether and numerous plant and animal natural products. In addition, epoxides often are important intermediates in the conversion of lipophilic aromatic and olefinic compounds into hydrophilic compounds which are more easily excreted. Since some of the above epoxides also may be toxic, mutagenic, and/or carcinogenic, there is wide interest in the enzymes which can convert them into polar and usually less reactive products. One group of these enzymes adds water to the epoxide to yield 1,2-diols. These enzymes are known as epoxide hydrolases (EC 3.3.2.3, formerly hydrases or hydratases) (1-4).

There now is excellent evidence for at least three epoxide hydrolases (EHs)[4] in

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[4] Abbreviations used: EH, epoxide hydrolase; TSO, *trans*-stilbene oxide; CSO, *cis*-stilbene oxide; BPO, benzo[*a*]pyrene 4,5-oxide; SO, styrene oxide; NBAP, 4-nitro-α-bromoacetophenone or *p*-nitrophenacyl-

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mammals: a predominantly microsomal form hydrating many xenobiotics (1-4), a microsomal enzyme hydrating the cholesterol epoxides and related compounds (5, 6), and a predominantly cytosolic form hydrating a variety of epoxides not on cyclic systems (4, 7-12). A cytosolic EH has been purified from several species by classical methods (9-12), while this laboratory has concentrated on affinity purification procedures (13).

There have been recent reports that several chemicals known to cause peroxisome proliferation, such as clofibrate, nafenopin, and di-(2-ethylhexyl)phthalate, also cause an increase in the activity of the predominantly cytosolic EH (14-17). Hammock and Ota (14) and Loury *et al.* (16) presented data supporting the hypothesis that the cytosolic EH activity in clofibrate-fed mice was due to the same enzyme present in control animals and this conclusion was supported further by recent purification studies (12, 13). It thus becomes important to compare the cytosolic EHs from clofibrate-fed and control animals rigorously to determine if the enzymes are, in fact, the same. For such a comparison to be convincing, it is important that yields of the enzyme from both normal and induced sources are essentially quantitative. To this end we have varied several parameters in the affinity purification procedure of the cytosolic EH (13) in order to produce high yields of the enzyme for these comparisons. The properties described here for the cytosolic EHs metabolizing *trans*-stilbene oxide (TSO) in cytosols from control and clofibrate-fed mice continue to support the hypothesis that these enzymes are indistinguishable.

EXPERIMENTAL PROCEDURES

Chemicals. Unless noted below, chemicals and solvents were from various chemical suppliers and were used without further purification. *Staphylococcus aureus* V8 protease was from Miles Laboratories (Naperville, Ill.) and all other biochemicals were from Sigma (St. Louis, Mo.). Clofibrate [ethyl α-(4-chlorophenoxy)-α-methylpropionate] was provided by Ayerst Laboratories and appeared identical to material synthesized previously in this laboratory using the method of Bach (18, 19). Radiolabeled *trans*- and *cis*-stilbene oxides (TSO and CSO, respectively) were prepared by [^{3}H]borohydride reduction of desyl chloride followed by treatment with base (20) (Fig. 1). Unlabeled benzo[*a*]pyrene 4,5-oxide (BPO) was provided by Dr. Richard Armstrong (Department of Chemistry, University of Maryland). Tritiated benzo[*a*]pyrene 4,5-oxide was provided by Dr. Michael McManus (then at NIH), and tritium labeled α- and β-cholesterol 5,6-oxides were provided by Dr. Alex Sevanian (Institute of Toxicology, University of Southern California). Unlabeled CSO was prepared by oxidation of the corresponding olefin with *m*-chloroperoxybenzoic acid. Unlabeled TSO, *cis*-stilbene, styrene oxide (SO), 1,1,1-

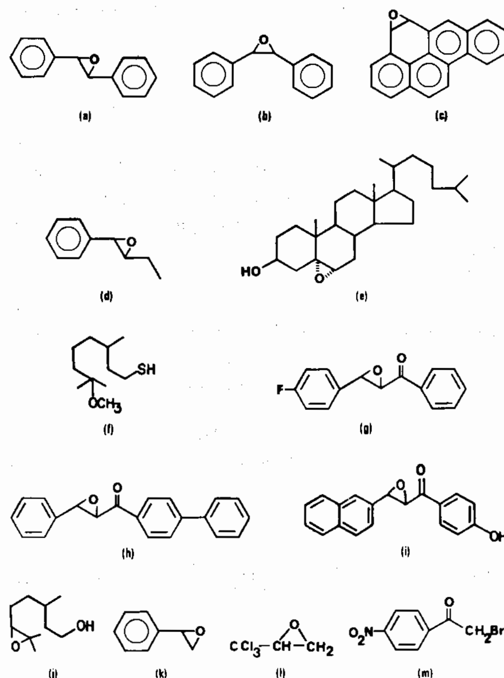

FIG. 1. Structures of substrates (a-e), affinity ligand (f), and inhibitors (g-m) used in this study. a, *trans*-stilbene oxide; b, *cis*-stilbene oxide; c, benzo[a]pyrene 4,5-oxide; d, *trans*-β-ethylstyrene oxide; e, cholesterol 5,6-oxide (α isomer shown); f, 7-methoxycitronellyl thiol; g, 4-fluorochalcone oxide; h, 4'-phenylchalcone oxide; i, 4-hydroxyphenylnapthalenyloxiranylmethanone; j, citronellol epoxide; k, styrene oxide; l, 1,1,1-trichloropropane oxide; m, 4-nitrobromoacetophenone.

bromide; LSC, liquid scintillation counting; MCT, 7-methoxycitronellyl thiol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing; 4-FCO, 4-fluorochalcone oxide; 4'-ϕCO, 4'-phenylchalcone oxide; TPCO, 1,1,1-trichloropropane oxide.

trichloropropane oxide (TCPO), tris (hydroxymethylamino)methane, 4-nitro-α-bromoacetophenone (NBAP), 1,4-butanediol diglycidyl ether, 1-hexanol, isooctane, were from Aldrich Chemical Company (Milwaukee, Wis.). Geraniol and citronellol 6,7-epoxides were prepared by epoxidation of the appropriate acetates in dichloromethane with *m*-chloroperoxybenzoic acid followed by base hydrolysis. Chalcone oxides were prepared by aldol condensation of the appropriately substituted aldehydes and acetophenones followed by conjugate addition of hydroperoxide anion as described earlier (21). Preparation of the 4- and 4′-azidochalcone oxides is described below (Fig. 2).

4′-Azidochalcone oxide. The 4-azidoacetophenone, mp 45–47°C, was prepared in 81% yield from treatment of 4-aminoacetophenone with 48% HBr and $NaNO_2$ in aqueous acetone (22). Condensation with benzaldehyde in the dark in ethanol containing 2 N NaOH for 1 h gave 64% of crude 4′-azidochalcone (recrystallized from ethanol, mp 94.5–96.5°C (23). Epoxidation by adding 1 N NaOH to 30% H_2O_2 and the chalcone gave a 69% yield of recrystallized (EtOH) 4′-azidochalcone oxide, mp 105–107°C.

4-Azidochalcone oxide. Reduction of 4-nitrochalcone oxide (21) with $SnCl_2 \cdot 2H_2O$ in conc HCl gave, after basic workup and recrystallization (95% EtOH), 4-aminochalcone oxide in 68% yield, mp 152–154°C (24). This product was stirred with 48% HBr and $NaNO_2$ in aqueous acetone and recrystallized (EtOH) to give the 4-azidochalcone in 64% yield, yellow needles mp 115–117.5°C (23). The 4-azidochalcone oxide, mp 80–81.5°C, was obtained in 51% yield from basic hydrogen peroxide in methanol. The high resolution mass spectrum of this new compound had a parent ion of *m/e* 265.0831, calcd for $C_{15}H_{11}N_3O_2$, 265.0852. NMR and ir data were also consistent with this structure.

Experimental animals. Clofibrate-treated, male, Swiss-Webster mice (25–30 g) received 0.5% (w/w) of the drug added to ground Purina rodent chow in 5 ml/100 g corn oil for 14 days while control animals received only the diet and oil. Cytosols and washed microsomes were prepared as previously described (14, 16, 17). The cytosol was either used immediately or frozen at −70°C (14). One freeze–thaw cycle has been found not to cause major changes in enzyme activities.

Protein and enzyme assays. Protein levels were estimated using a previously described modification (17) of the method of Bradford (25). Data were collected and analyzed using an Atari 400 microcomputer interfaced with a Gilford Manual EIA reader.

Several substrates were used to monitor EH activity. Since TSO has previously been shown to be selectively hydrated by the cytosolic enzyme (7, 26, 27), it was used in a partition assay to monitor cytosolic EH activity (20, 28, 29). CSO, in contrast, is hydrated at only 3% the rate of TSO by purified murine cytosolic EH at pH 7.4 (the optimum pH for this form) (13). BPO metabolism is not detectable with cytosolic EH (26, 27). Periodically the aqueous phase of these assays was analyzed further by extraction with hexanol or development on thin-layer chromatography (TLC) plates with a diol standard to ensure that the diol was the only polar metabolite produced.

The microsomal EH, at its pH optimum (ca. 9.0), rapidly hydrates CSO and BPO at similar rates, while TSO metabolism by this enzyme is insignificant relative to the cytosolic enzyme. Thus, rates of hydrolysis of TSO and BPO were used as indicators of activity of classical cytosolic EH and microsomal EH, respectively, regardless of the fraction in which the activity occurred. CSO hydration was corrected for the contribution by both microsomal and cytosolic EH in mouse liver (16).

For kinetic studies, the hydrolysis of TSO was analyzed by the spectrophotometric method of Hasagawa and Hammock (30) using substrate concentrations ranging from 1.67×10^{-3} to 1×10^{-1} mM. The decrease in absorbance was monitored for at least 5

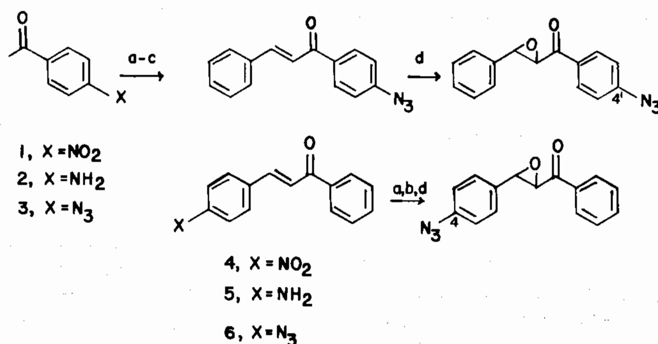

FIG. 2. Synthetic routes to 4′-azidochalcone oxide (top) and 4-azidochalcone oxide (bottom). Reagents: (a) $SnCl_2$, conc HCl; (b) 48% HBr, $NaNO_2$, acetone; (c) PhϕCHO, C_2H_5OH, 2 N NaOH; (d) H_2O_2, 1 N NaOH.

min at 229 nm using a Varian Cary 219 spectrophotometer interfaced with an Apple IIe computer. Kinetic parameters were calculated both from least-squares analysis of Lineweaver–Burk plots (31) and by Wilkinson's method (32) which yielded essentially identical results.

Hydration of BPO at a final concentration of 5 $\times 10^{-5}$ M was monitored by a slight modification (4, 13) of the method of Jerina *et al.* (33). Care was taken to minimize chemiluminescence. Hydrolysis of the α- and β-cholesterol epoxides previously was shown to be insignificant in the cytosol of mice (Sevanian and Hammock, unpublished). Thus, a slight modification of the above procedure was used to monitor hydration of these substrates using purified cytosolic EH (29, 34). A final concentration of 5 $\times 10^{-5}$ M was used for these substrates, approaching the limit of their solubility at 6 $\times 10^{-5}$ M (34).

Cytosolic glutathione (GSH) S-transferase activity was assayed using CSO at 5 $\times 10^{-5}$ M in pH 7.4 phosphate buffer with 5 mM GSH in a manner similar to that used for the cytosolic EH except that the reaction was terminated by the addition of hexanol. Hexanol extracts both the diol and the epoxide from the radiolabeled conjugate which remains in the aqueous phase (20). At least three replicates were run for each reported data point. In general, data were analyzed as described previously by this laboratory (20, 28, 29).

Inhibition of TSO hydrolysis using crude cytosol and purified cytosolic EH. For these studies the cytosols or pure proteins were diluted in 76 mM sodium phosphate buffer (pH 7.4) to yield similar initial rates of TSO hydrolysis (4–7 nmol/min/ml depending upon replicate but within 1 nmol/min/ml for the pair of enzymes being compared). Aliquots of 100 μl were taken for assay. Inhibitors were added to the enzyme in 1 μl of ethanol. Following a 10-min preincubation at 37°C, 1 μl of ethanol containing tritiated TSO (5 $\times 10^{-5}$ M final concn) was added, and the assay was carried out as described above. Data were expressed as a percentage of the activity in incubations receiving ethanol alone and each point represents an average of triplicate determinations of replicates using at least two different enzyme preparations.

Dialysis. For initial studies on the suitability of various inhibitors to be used in affinity chromatography, the cytosol fractions from control and clofibrate-treated mice were diluted in 76 mM sodium phosphate buffer (pH 7.4). Four milliliters of the enzyme solution and 40 μl of the appropriate inhibitor diluted in ethanol (to give 80–95% inhibition) were preincubated for 15 min at 37°C. The samples were added to 6.4-mm diameter, washed, Spectrapor (Los Angeles, Calif.) dialysis sacks, and identically but separately dialyzed against 800 ml of sodium phosphate buffer at 4°C. At various times during dialysis, three 100-μl aliquots were removed and assayed for cytosolic EH activity using 5 $\times 10^{-5}$ M TSO. Buffers were changed at 2, 6, 10, and 22 h. Alternatively, 0.5 ml of a 4× concentrated solution of various inhibitors in 76 mM sodium phosphate buffer at pH 7.4 was added to 1.5 ml purified cytosolic EH and the fractions dialyzed as above.

Preparation of affinity column. The affinity matrix was prepared essentially as described previously (13). To Sepharose CL-6B (washed with 10 vol of distilled water) was added 28 ml of 1 N NaOH containing 57 mg of $NaBH_4$ and 5.7 ml of 1,4-butanediol diglycidyl ether. The slurry was swirled at room temperature for 8 h using an orbital shaker. The resulting epoxy-activated resin was washed with 10 vol each of water, 1/1 methanol–water/methanol, 1/1 methanol/water and suction-filtered to remove most liquid. Analysis of the free epoxy functionality with sodium bisulfite routinely yielded 5–15 μeq/g Sepharose (wet wt) (35).

The affinity ligand was prepared by organomercuration-demercuration of citronellol acetate, conversion to the thiol acetate by the Mitsunobu procedure (36), and reduction of the free 7-methoxycitronellyl thiol (MCT) with $LiAlH_4$ (37) (Fig. 1). Approximately a four-fold excess of MCT in methanol was added to the epoxy-activated matrix in 1/1 methanol/water containing 0.05 M $NaHCO_3$. After swirling for 24 h, the gel was exhaustively washed and stored at 4°C in absolute ethanol containing ca 0.2% butylated hydroxyanisole. Before loading, the gel was exhaustively washed with the 76 mM sodium phosphate buffer [pH 7.4, 0.1 mM sodium EDTA, 0.1 mM dithiothreitol (DTT)] which was subsequently used for loading, washing, and eluting with several additives.

Affinity purification of cytosolic EH. A variety of procedures were used to optimize the affinity purification of the cytosolic EH using the MCT gel. Optimization procedures were carried out at 2 ± 0.2°C using a 1.5- to 2.5-ml bed volume in 8-mm i.d. Wheaton reflux condensers serving as coolant-jacketed mini-columns. Cytosol was diluted to 2% (original liver wt/v) in the EDTA, DTT, phosphate buffer described above and passed through the MCT–Sepharose column at 0.2–0.3 ml/min using a peristaltic pump. Following a column wash using a volume of buffer at least equal to the volume of cytosol loaded on the column several elution regimens were evaluated by adding various reagents to the wash buffer at the above flow rates. Fractions were assayed as described above for TSO, CSO, and BPO hydrolysis as well as GSH conjugation.

Preparative studies were carried out at 2–4°C using the column described above. After washing the column (8-ml bed vol) with 700–1000 ml of buffer at 0.7–0.9 ml/min, it was immediately loaded with hepatic cytosol diluted at 2% original wet wt/v from control (1200-ml total) or clofibrate (300–350 ml)-treated mice at 0.5–0.58 ml/min. The column was subsequently washed with approximately 1 liter of buffer at 0.8–0.9 ml/min, then cytosolic EH was then eluted with 45–50 ml of a saturated solution of 4-azidochalcone

oxide in buffer. Following protein analysis, samples containing chalcone oxide were dialyzed by one of several procedures discussed below and all samples analyzed as above. Alternatively, the column was eluted with 5×10^{-4} M 4-fluorochalcone using the above procedure.

Gel permeation. A Sephacryl S-300 column (1.6 × 85 cm, Pharmacia) was poured and equilibrated with 50 mM sodium phosphate buffer, pH 7.5, 0.1 mM EDTA, and 0.02% sodium azide then calibrated with Blue Dextran, bovine serium albumin, ovalbumin, myoglobin, and cytochrome *c.* Cytosolic EH samples [10% cytosol from control mice (4.3 mg protein/1 ml), 10% cytosol from clofibrate-fed mice (6.8 mg protein/0.5 ml), affinity purified control cytosolic EH (76 μg protein/ml), affinity purified clofibrate-induced cytosolic EH (58 μg protein/ml), and the latter sample mixed with calibration proteins] were loaded on the column in separate but sequential runs. Elution was carried out at 4°C at a flow rate of 5 ml/h. Fractions (2.5 ml) collected were assayed for TSO hydrolytic activity and protein content.

Gel electrophoresis. Discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (38) with a Hoefer Vertical slab gel system (SE 600, Hoefer Scientific Instruments). Molecular markers included phosphorylase *b* (97,000), bovine serum albumin (68,000), ovalbumin (43,000), soybean trypsin inhibitor (21,000, 20,000), and cytochrome *c* (13,000). Gels were stained with 0.1% Coomassie brilliant blue in ethanol/water/acetic acid (33/11/3.6) and destained in the same solvent. Under these conditions, a detection limit of less than 1 μg/band was obtained. A 12 or 12.5% gel was used for molecular weight determination and a 15% gel was used for protease digestions and peptide mapping with a 4% stacking gel in each case.

Isoelectric focusing. Isoelectric focusing (IEF) was performed with a LKB horizontal polyacrylamide gel apparatus (LKB 2117 Multiphor System). Wide range gels used pH 3.5 to 9.5 or pH 5 to 8 Ampholite (LKB), while narrow range gels were run with pH 4 to 6 in 4.8% polyacrylamide gels. Evans blue (pH 4.5) was used as the tracking dye. After electrofocusing, lanes spotted with cytosolic EH were cut into 0.5-cm segments and incubated overnight at 4°C in 1 ml of distilled water. The next day, pH and TSO hydrolytic activity of the supernatant were measured. Gels were also fixed with a 3.5% sulfosalicyclic acid and 11.5% trichloroacetic acid solution, and then stained with Coomassie brilliant blue.

Peptide mapping. Peptide mapping was performed according to the method of Cleveland *et al.* (39). Briefly, 30 μl of affinity purified protein was denatured by boiling for 5 min in sample buffers (0.125 M Tris · HCl, pH 6.8, 1% SDS, 1 mM EDTA, and 3% β-mercaptoethanol). After cooling, the samples were loaded into the wells of a 15% SDS-PAGE gel, and 3 μg of respective protease was added. After the materials were stacked in the stacking gel, the power was turned off for 30 min and then electrophoresis resumed until the tracking dye was 1 cm from the bottom of the slab. Proteases used included DPCC-treated trypsin, α-chymotrypsin, and *S. aureus* V8 proteases. For non-denatured protease digestions, 30 μg of the enzyme (in 25 mM phosphate buffer, pH 7.8) was digested with 1/100 (w/w) α-chymotrypsin, added twice for 2 h at 37°C. At the end of the incubation, the protein was boiled in the sample buffer to inactivate the protease and then run on a 15% SDS-PAGE gel. Amino acid analyses were performed on the cytosolic EH by A. Smith of the University of California, Davis, peptide laboratory following electrophoretic elution of the affinity purified material from SDS-PAGE (40).

Western blotting. Rabbit anticytosolic EH antiserum was raised in New Zealand female white albino rabbits (Batton and Kingman, Fremont, Calif.) by three inoculations with the 60 kDa affinity purified cytosolic EH in Freund's complete adjuvant (1/1 vol/vol) using the general procedure of Vaitukaitis (41). Six weeks later, antiserum was collected 10 days after two additional boosts.

Western blots were carried out with affinity column purified cytosolic EH from control and clofibrate-fed mice. After separation on SDS-PAGE, proteins were transferred onto nitrocellulose membrane with a trans-blot apparatus (Bio-Rad) at 30 V/100 mA. Immunoblot was performed according to the procedures specificed by Bio-Rad using the rabbit anticytosolic EH antiserum (1/50) and a goat anti-rabbit IgG horseradish peroxidase conjugate from Bio-Rad (1/2000).

RESULTS AND DISCUSSION

Development of elution systems for MCT Sepharose. It was demonstrated previously that MCT Sepharose is capable of retaining the cytosolic EH activity for TSO present in a large volume of mouse cytosol while most of the GSH transferase activity passes through the column (13). Since most of the substrates and inhibitors of the cytosolic EH, including MCT, are lipophilic, the hypothesis was developed that the enzyme–column interaction largely was hydrophobic. Thus, a step gradient of the nonionic detergent, Lubrol-PX, was used. The cytosolic EH activity eluted between 0.3 and 1.0% detergent with a 60% recovery of enzyme activity. However, 40% of the protein applied to the column also eluted in this fraction resulting in only a twofold purification with a sp act of 7.4 nmol/min/

mg protein using TSO as substrate. SDS-PAGE (not shown) confirmed the presence of numerous contaminating bands. Although improved loading techniques have reduced the apparent nonspecific binding of cytosolic proteins to the MCT gel, a more specific elution was required to obtain homogeneous enzyme. However, these data indicate that moderate concentrations of ethoxylate nonionic surfactants such as Lubrol-PX (0.1%) or Triton X-100 can be used to wash the column following loading or after biospecific elution of the cytosolic EH.

pH profiles were very similar to cEH activity in normal and clofibrate cytosols as well as purified enzymes (data not shown). The activity plateaued in each case near pH 7.4 with the rates of hydration at pH 5 and 9 only about 40 and 20% of that observed at 7. The cytosolic EH loses catalytic activity when held at high pH and then returned to 7.4 for assay, but as expected for a sulfhydryl enzyme, the stability of the enzyme actually is enhanced at low pH. Thus, it might be possible to elute the cytosolic EH from MCT Sepharose by reducing the pH of the eluting buffer. Nevertheless, as shown in Fig. 3A, a step gradient to pH 5.0 failed to elute the cytosolic EH although some additional GSH transferase activity did elute from the column. Subsequent elution with 4-azidochalcone oxide as described later indicated that the cytosolic EH activity was not denatured extensively by exposure to low pH while bound to the MCT-Sepharose. pH changes could be used to remove other contaminating proteins from the MCT column. This information may indicate that the low activity of the cytosolic EH at acidic pH is due to reduction in turnover rather than binding. One could argue along this line that catalytic activation of water would be difficult at low pH or that solution protonation competes with electrophilic activation at the catalytic site (21, 42).

Alternatively, the ligand itself or a substrate could be used for elution (Fig. 1). Although they are excellent substrates, fatty acid, and other 1,1-disubstituted epoxides are known to be turned over very rapidly by the cytosolic EH (43, 44) and would likely be degraded in the process of eluting the enzyme. In contrast, a variety of trisubstituted terpenoid epoxides have been found to be turned over more slowly, to have lower K_m's in kinetic studies than similar 1,1-disubstituted epoxides, and to possess high apparent affinities as indicated by inhibition of the enzyme (21, 27, 43). Thus, gradients of citronellol epoxide or geraniol 6,7-epoxide were used for elution as shown in Fig. 3B. Such compounds have surfactant properties, but a recovery of over 80% of the loaded cytosolic EH activity at concentrations far below those used with Lubrol indicates bioselective elution. This column gave a 30-fold purification with recovery of 80% of the cytosolic EH activity at a sp act of 100 nmol/min/mg protein. However, several other minor proteins and a major protein with an apparent molecular weight of 30,000 also are eluted with these terpenoid epoxides in addition to the 60,000 M_r band for the cytosolic EH as shown by SDS-PAGE. This coelution may be caused by the similarity of the terpenoid epoxides to MCT. Whether these proteins are important in steroid and terpene metabolism remains to be determined. As with the previous column, subsequent elution with 4-azidochalcone oxide (see below) and Lubrol-PX failed to elute additional cytosolic EH, although the Lubrol did elute additional protein.

A number of inhibitors were examined as possible ligands for the elution of the cytosolic EH from the MCT gel. Several properties must be considered for successful elution. Certainly, reversibility is required if one wishes to recover enzyme activity. This requirement eliminates a variety of divalent cations and sulfhydryl reagents such as NBAP and organomercurials (21, 27). At the millimolar concentrations normally used for analysis of the microsomal EH, styrene oxide is not a good substrate for the cytosolic EH (8); however, it is hydrolyzed faster in mouse cytosol than microsomes at lower concentrations (21, 27). Mullin and Hammock (21) argued that styrene oxide acts to block an essential sulfhydryl group in the enzyme and possibly could be considered a suicide substrate. The inhibition of the cytosolic

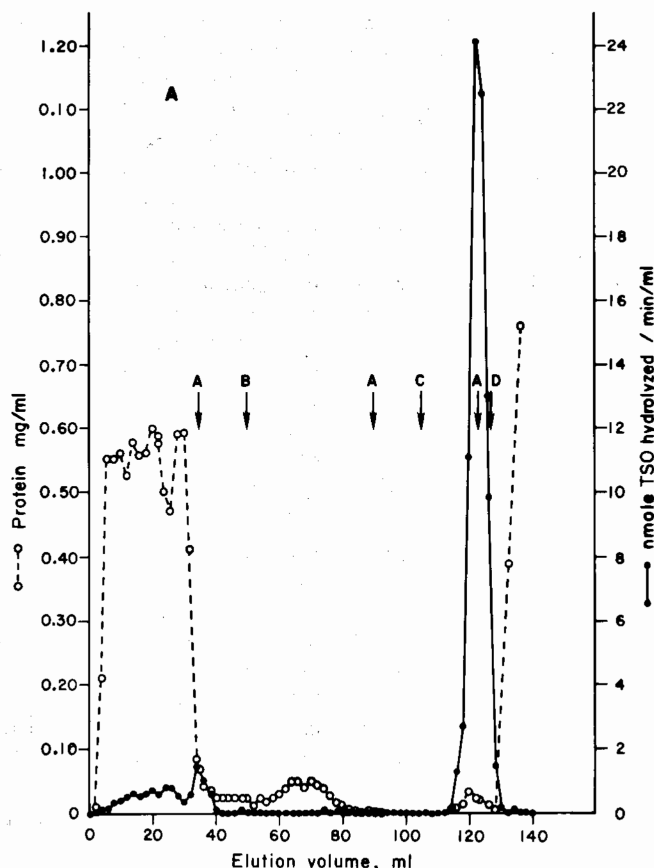

FIG. 3. Affinity purification of cytosolic EH from control mouse liver cytosol. Fig. 3A shows elution with a chalcone oxide. Elution of Protein (O) and cEH (●) from MCT Sepharose: A, 76 mM sodium phosphate buffer, pH 7.4; B, pH step gradient, 6 ml each of pH 7.0, 6.5, 6.0, 5.5, 5.0; C, 4-azidochalcone oxide, 5 ml each of 1×10^{-5} M, 1×10^{-4} M, 1×10^{-3} M; D, Lubrol-PX, 0.3% (5 ml), 1.0% (5 ml). This column gave a 92-fold purification of the cytosolic EH in 57% yield with a specific activity of 356 nmol/min/mg protein following dialysis to remove the chalcone oxide. Fig. 3B shows elution with a terpene oxide. Elution of protein (O) and cytosolic EH (●) from MCT Sepharose: A, 76 mM sodium phosphate buffer, pH 7.4; B, Lubrol-PX, 0.1%; C, citronellol epoxide, 10 ml each of 1×10^{-5} M, 1×10^{-4} M, and 1×10^{-3} M; D, 4-azidochalcone oxide, 5×10^{-4} M.

EH by styrene oxide is time dependent and appears irreversible under the conditions employed in this study, rendering it a poor substrate for elution of enzyme activity (Fig. 4).

Mullin and Hammock (21) reported that some substituted chalcone oxides were potent, reversible inhibitors of the cytosolic EH. Although some of the more potent compounds in this series appear to be slow, tight binding inhibitors, time courses of inhibition indicate that maximal inhibition is obtained within minutes, which is adequate for use in affinity chromatography. It would be advantageous to use chalcone oxides which are potent inhibitors and thus have a high affinity for the enzyme. However, some of the most potent compounds such as 4 and 4′-phenylchalcone oxide (Fig. 1, I_{50}'s 6×10^{-8} M, 8×10^{-7} M) display some limitations. These compounds cause slow and incomplete recovery of enzyme activity upon dialysis. This may reasonably arise from their lipophilicity which leads to poor water solubility and tighter binding. To enhance water solubility, a phenolic deriv-

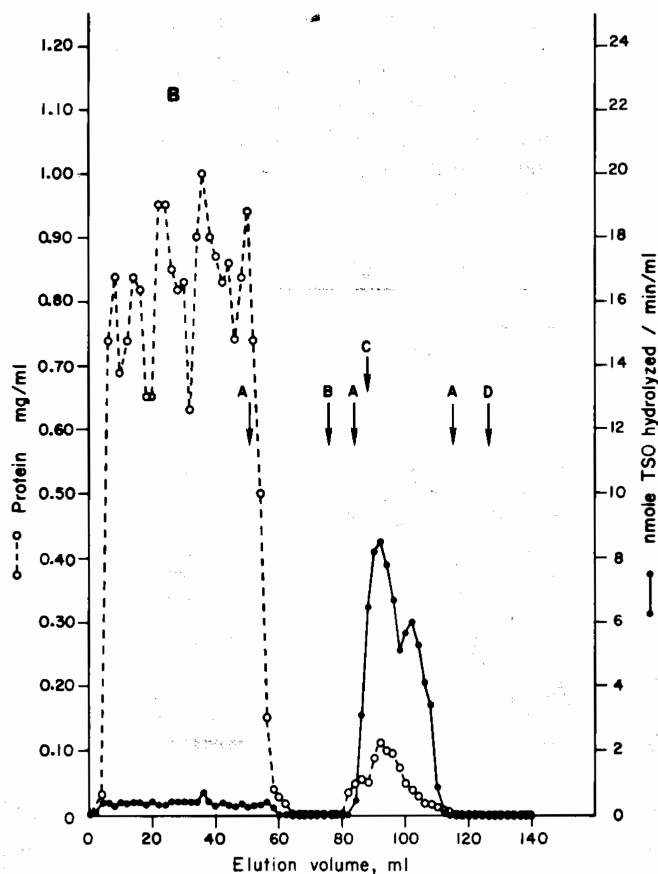

FIG. 3—*Continued.*

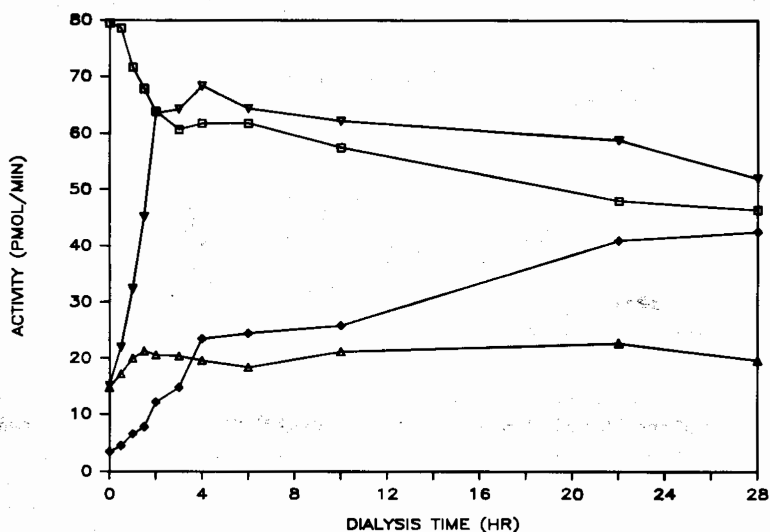

FIG. 4. Reversibility of inhibition of the cytosolic EH by selected compounds: ethanol control (□), 4-fluorochalcone oxide (1×10^{-5} M, ▽), 4-phenylchalcone oxide (1×10^{-6} M, ◇), and styrene oxide (2.5×10^{-3} M, △). Methods as described in text.

ative (Fig. 1, 4-hydroxyphenyl naphthalenyloxiranyl methanone, I_{50} 8×10^{-7} M) was successfully used to elute the cytosolic EH. The enzyme appeared homogeneous on SDS-PAGE, but complete removal of the inhibitor remained time consuming. The approach of using low concentrations of very potent, but somewhat water soluble inhibitors may ultimately prove successful, however, other approaches were used in the short term.

As shown in Fig. 4 and by dialysis studies, total activity from cytosolic EH inhibited with slightly less potent compounds

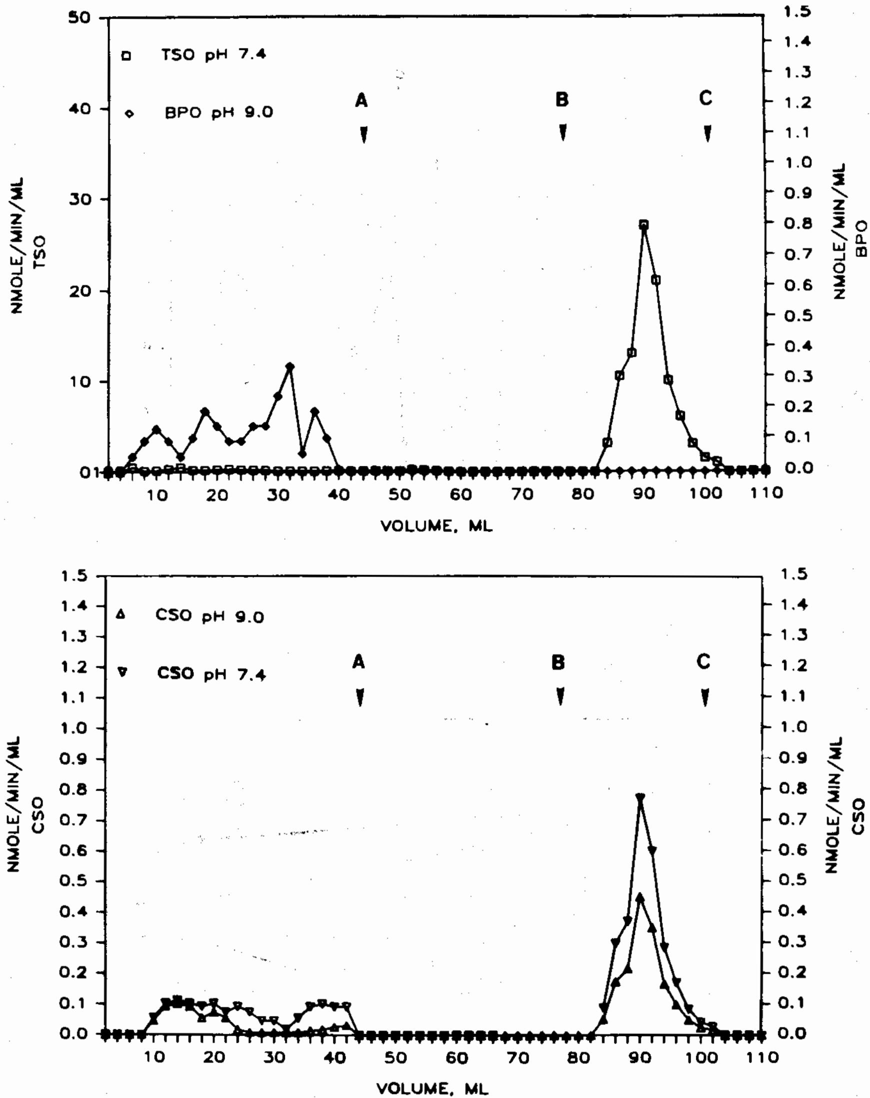

FIG. 5. Elution of epoxide hydrolase activities from paired MCT gels using cytosols from control (left two panels) and clofibrate-fed mice (right two panels). In both cases the upper graphs show the hydrolytic activity on TSO at pH 7.4 (□) and BPO at pH 9 (◇) while the lower two graphs indicate the hydrolysis of CSO at pH 7.4 (▽) and pH 9.0 (△). Note that the scale for hydrolysis of TSO is different from other substrates. In each case 60 ml of 2% (original wet wt/vol) cytosol freshly prepared from control or clofibrate-fed mice was pumped onto equivalent 2.5-ml beds of MCT–Sepharose. The columns were eluted with the following regime: A, wash with buffer; B, elute with 1 mM 4-azidochalcone oxide; C, wash with buffer.

such as the 4-fluoro, 4-azido, or 4′-azido chalcone oxides (I_{50}'s 2×10^{-6}, 1.6×10^{-6}, and 4.8×10^{-6} M, respectively) could be recovered with shorter dialysis times. Ultimately, 4-azidochalcone oxide was used in routine studies because it was somewhat more water soluble, has a moderate I_{50}, and its removal can be monitored spectrophotometrically (Fig. 3, 5, Table I). The azidochalcone oxides were prepared as photoaffinity labels for the cytosolic EH and are potentially reactive. Also, even trace levels of these compounds may cause analytical problems following Edman degradation of peptides. Thus, for some studies 4-fluorochalcone oxide was used for elution. Careful dialysis can result in equivalent, essentially quantitative yields of cytosolic EH following elution from MCT Sepharose with either 4-fluoro or 4-azidochalcone oxide.

Recovery of cytosolic EH activity. By simply diluting the effluent from the MCT column, cytosolic EH activity can be detected with TSO even in the presence of chalcone oxides. This property is of obvious

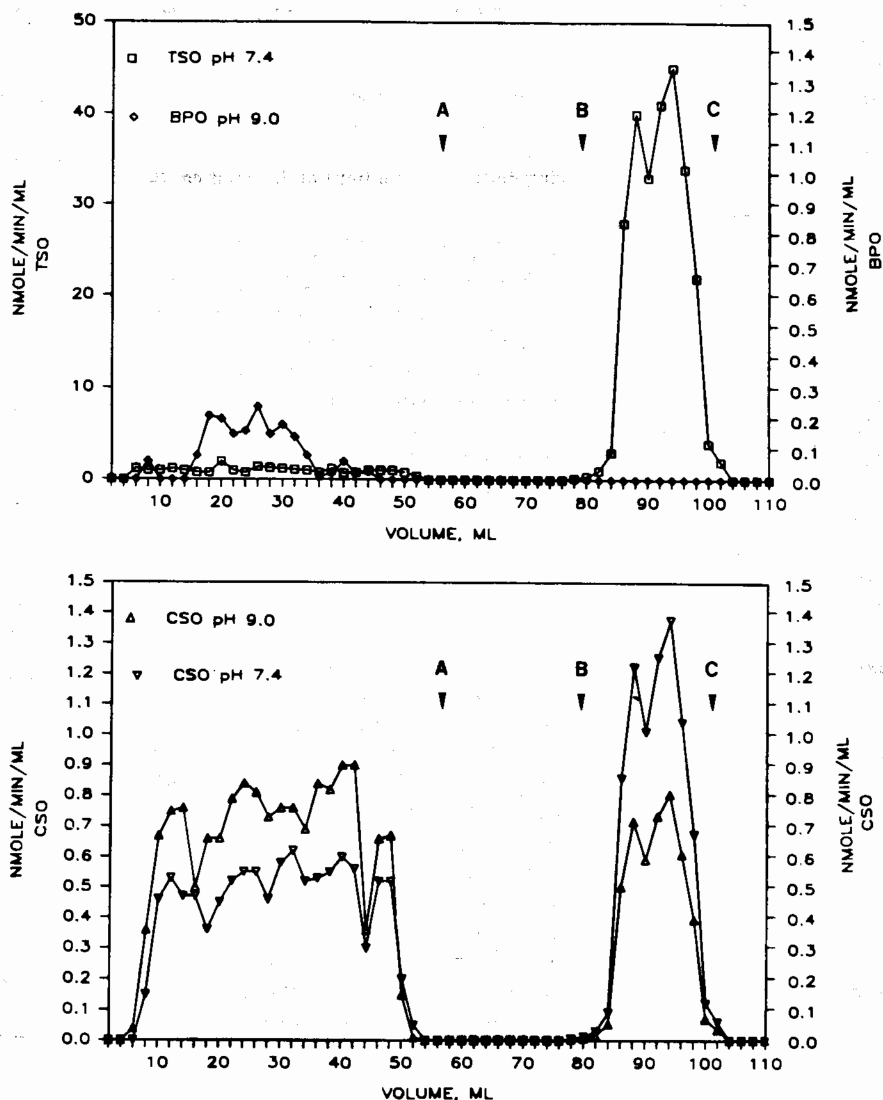

FIG. 5—*Continued.*

TABLE I

RECOVERY OF PROTEIN AND ENZYME ACTIVITIES FOLLOWING PREPARATIVE AFFINITY PURIFICATION OF CYTOSOLIC EPOXIDE HYDROLASE[a]

	Protein (mg)		TSO hydrolysis pH 7.4		CSO hydrolysis pH 7.4		BPO hydrolysis pH 9.0
	Control	Clofibrate	Control	Clofibrate	Control	Clofibrate	Clofibrate
Loaded	1596	419	5011	5712	316	528	146
Eluted with 4-ACO	3.55	4.73	2290	4440	70	112	ND[b]
Percentage recovered	0.22	1.13	46	82	20	21	0
Specific activity (loaded)	—	—	3.14	13.4	0.20	1.26	0.35
Specific activity (eluted)	—	—	645	676	19.8	23.7	ND[b]
Purification	—	—	205	50	99	19	—

[a] Activities loaded and eluted are expressed in units defined as nmol/min while specific activities are nmol/min/mg protein. Control (single experiment) and clofibrate (average of two experiments) cytosols are described in Methods.

[b] No BPO hydrolysis was detected following dialysis with a limit of detect of ca. 10 pmol/min/mg protein.

benefit in the optimization of column conditions, however, it is not adequate for the recovery of high levels of enzyme activity. The EH activity in crude cytosol is very stable so long as divalent cations are excluded. However, the homogeneous enzyme is moderately unstable over long periods. Thus, some care must be taken with dialysis procedures. Thus, a variety of factors were examined resulting in the obvious inclusion of EDTA and DTT during dialysis. Inclusion of BSA as a carrier obviously increases the recovery of enzyme activity.

Comparison of cytosolic EHs in cytosolic and apparently homogeneous preparations from control and clofibrate-fed mice. For subsequent studies on the regulation of EH activity, it is important to rigorously test the hypothesis that the EH activity on TSO in the cytosols of control and clofibrate-fed mice is due entirely to the same, single isolated enzyme. Similarities in substrate selectivity, comigration on analytical isoelectric focusing, and apparently identical R_f's on SDS electrophoresis provided a strong indication that the cytosolic EH from liver (and in some cases kidney) of control and clofibrate-treated mice were identical (13, 14, 16). However, several additional pieces of evidence for this hypothesis are presented here. Good evidence comes from the MCT Sepharose column itself. Based upon over 20 separate experiments using a variety of loading and elution techniques, the TSO hydrolytic activities in the cytosols of control and clofibrate-fed animals behave similarly on the column and upon subsequent SDS-PAGE. The enzyme activities elute with similar structures and concentrations of ligands (Fig. 5) and, as shown in Table I, the recovered specific activities are very similar. Since the specific activities observed on TSO are much lower in control cytosol, one does not expect equivalent purification factors. Similarly, a perfectly controlled experiment in which equal amounts of protein and enzyme activity are applied to the MCT Sepharose column cannot be run. However, a series of experiments in which the amount of protein and amount of activity loaded vary further indicate the similarity of activities in control and clofibrate cytosols. Similar stabilities of the affinity purified cytosolic EHs from livers of control and clofibrate-fed mice during dialysis provide additional evidence for identity.

It has been noted previously that clofibrate increases cytosolic EH activity with

either TSO, TESO, or CSO used as substrate (14, 16, 17). Thus, the relative activities of crude and purified cytosolic EHs from control and clofibrate-fed animals for different substrates provide additional evidence of similarities. For instance, the data in Table I indicate that the hydrolysis of CSO relative to the hydrolysis of TSO is very similar in the affinity purified (eluted) fractions from control and clofibrate-fed mice (3.1 and 3.5%, respectively). Similar trends can be seen as one compares eluted TSO activity with CSO activity run at both 7.4 and 9.0 from control and clofibrate-fed mice in Fig. 5.

Specific activities of over 1400 nmol/min/mg protein are obtained with TSO as substrate for affinity purified cytosolic EH. Hammock and Hasagawa (27) pointed out that TESO is hydrated over 16 times faster in mouse cytosol than is TSO. This relative activity on TSO and TESO is borne out with purified enzyme from control and clofibrate-treated animals (22× and 15×, respectively). With *trans*-β-propyl styrene oxide hydrated 32 times faster than TSO, and other lipid epoxides hydrated still faster, one can anticipate velocities of the cytosolic EH of well over 50,000 nmol/min/mg protein for appropriate substrates. This indicates that the cytosolic EH of mouse liver can turn over substrate more than 10 times faster than the microsomal EH acts on good substrates such as phenanthrene 9,10-oxide and roughly 100 times faster than mediocre substrates such as styrene oxide and BPO (45). This specific activity of the cytosolic EH is similar to that of the affinity purified microsomal EH from *Spodoptera eridania* (46, 47). Previous studies have shown that epoxides on cyclic systems such as cyclohexane oxide and BPO are very slowly metabolized in the cytosolic fraction of mouse liver (26, 27). Figure 5 and Table I indicate that under routine assay conditions, no hydrolysis of BPO is detected in the affinity purified fraction from control or clofibrate-fed mouse liver cytosols. Even under very rigorous conditions using highly active, pure cytosolic EH, it was not possible to demonstrate that enzymatic hydration of BPO or the cholesterol epoxides were different from background levels. These data indicate that such substrates are useful for measuring other epoxide hydrolase activities in the presence of the cytosolic EH.

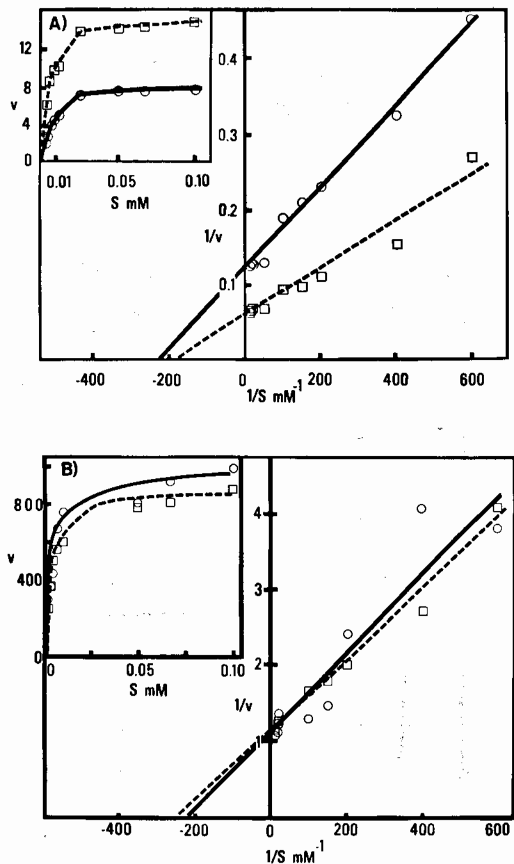

FIG. 6. Kinetic analysis of the hydrolysis of TSO by crude cytosol (top panel) and affinity purified cytosolic EH (bottom panel) from control and clofibrate-fed mice. In each case the solid line represents data from control (—) and the dotted line data from clofibrate-fed mice (---). The dependence of initial velocities on substrate concentration is shown in the insert (upper-left-hand corner) while the large graphs are least-squares fitted Lineweaver–Burk plots. The data points were obtained from four different protein concentrations for the cytosol (control 73, 108, 146, 216 μg and clofibrate 79, 148, 158, and 296 μg protein added) and the affinity purified cytosolic EH (0.66 to 0.94 μg) indicating that velocities were linearly dependent on protein concentration. Kinetic constants (V_{max} is nmol/min/mg protein and $K_{m,\ app}$ as μmol) were determined as described by Wilkinson (31) and are as follows for control cytosol (8.4 ± 0.23, 4.75 ± 0.51), clofibrate cytosol (15.8 ± 0.4, 4.13 ± 0.41), purified control (964 ± 75, 4.61 ± 1.18), and purified clofibrate (847 ± 21, 3.71 ± 0.37).

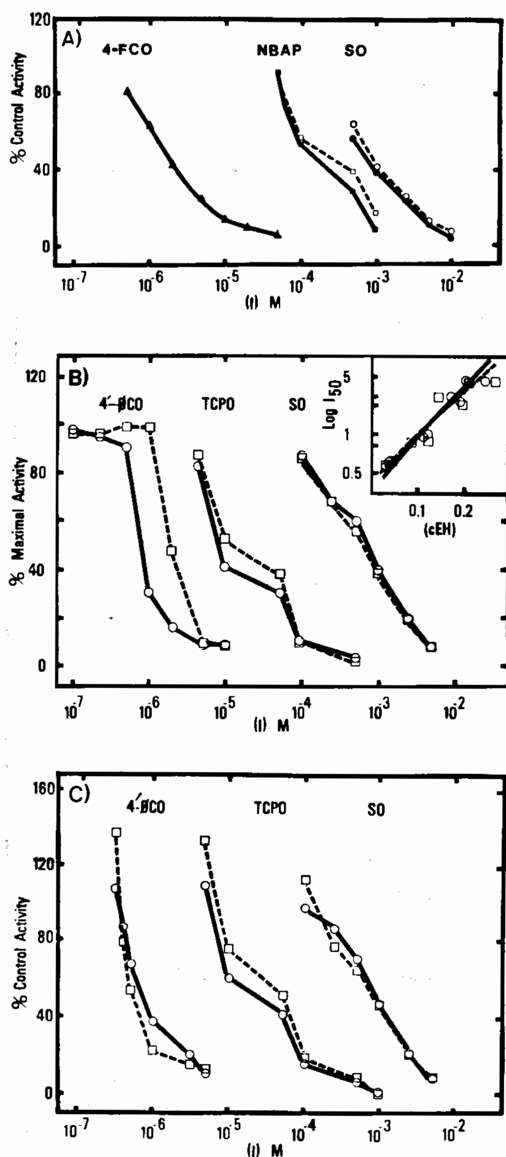

FIG. 7. A comparison of several compounds as inhibitors of TSO hydrolysis by crude cytosol (top two panels) and affinity purified cytosolic EH (bottom panel) from control and clofibrate-fed mice. In each case the solid line represents data from control and (—) the dotted line data from clofibrate-fed mice (---). Compounds include 4-fluorochalcone oxide (4-FCO, note control and clofibrate data are not distinguishable), 4-nitrobromoacetophenone (NBAP, data from purified enzyme are similar), styrene oxide (SO), 4'-phenylchalcone oxide (4'ϕCO) and 1,1,1-trichloropropane oxide (TCPO). Hydrolysis of TSO is presented as a percentage of maximal activity vs the log of the final inhibitor concentration. The insert shows the dependence of log I_{50} for 4'-ϕCO on the initial activity of the cytosolic EH (nmol/min/mg protein). All reactions were performed in triplicate and the values shown are the mean of two separate experiments using cytosol from different mice.

In addition to initial rates, the kinetics of hydrolysis of TSO appear similar. Of special note, the K_m's are essentially identical in cytosol and affinity purified preparations from both control and clofibrate-fed mice. The V_{max} was increased in crude cytosol from clofibrate-treated mice, but this difference was eliminated when purified enzymes were compared (Fig. 6).

In addition, inhibitors can be used to illustrate the similarity of the enzymes. For instance, in Fig. 7, the inhibition of cytosolic EH activity in crude cytosol and affinity purified preparations is shown. The close similarity of the lines from the two preparations is surprising for styrene oxide and NBAP since these are rather general in their ability to inhibit enzymes with a catalytically active thiol (Fig. 7). A definite plateau is observed with high micromolar concentrations of NBAP with both the crude and pure preparations. The variety of possible explanations for this phenomenon have not yet been experimentally tested.

Figure 7 further illustrates that similar experiments can be applied to the affinity purified enzymes. In this case, for example, three structurally diverse epoxides were used as inhibitors of enzyme activity. Styrene oxide is hydrolyzed faster by the crude cytosol of mouse liver than by the microsomal fraction when incubated at low substrate concentration (27), while at high substrate concentrations, it can irreversibly inhibit the enzyme (Figs. 4, 7) (21, 27). 1,1,1-Trichloropropane oxide is a potent inhibitor of the microsomal EH (1) but weak inhibitor of the cytosolic EH (21). Inhibition appears also to be reversed as the inhibitor is hydrated. 4'-Phenylchalcone oxide is the most potent inhibitor yet reported for the cytosolic EH possibly acting as a transition state mimic. The inset in Fig. 7 indicating that the potency of 4'-ϕCO varies with the concentration of the enzyme is an indication of near stoichiometric inhibition which often is true of transition state inhibitors. Inhibition slowly de-

creases with time as the chalcone oxide is metabolized (21). The similarity of inhibition regression lines in each case provides further proof for the hypothesis that enzymes from control and clofibrate-treated animals are identical.

A variety of biochemical parameters further indicate similarity. The pH optima for cytosolic EH activity from control and clofibrate-fed animals are similar. The pH optimum for the homogeneous enzyme also is at pH 7, but the curve is much sharper than for cytosolic activities. Figure 8 demonstrates that the molecular weights associated with TSO hydrolytic activity are indistinguishable by gel permeation on Sephacryl S300 from crude and affinity purified fractions. The apparent molecular weight of the affinity purified forms remained identical when they were mixed with protein molecular weight standards before chromatography with hydrolytic activity on TSO eluting just after bovine serum albumin. These data indicate that the monomeric (59 kDa SDS, 55 ± 5 kDa gel permeation) as well as the dimeric forms (48) of the enzyme retain catalytic activity.

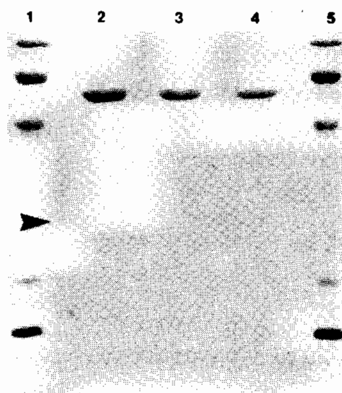

FIG. 9. SDS-PAGE gel of affinity purified cytosolic EH from clofibrate-fed mice. Tracks 1 and 5 contain standards (97, 68, 43, 21, 20, and 13 kDa) while tracks 2, 3, and 4 contain 4, 2.5, and 1 μg of cytosolic EH. The arrow indicates a minor band at 29.5 kDa. On this same gel 0.05 μg of cytosolic EH could be detected as a faint band with Coomassie blue.

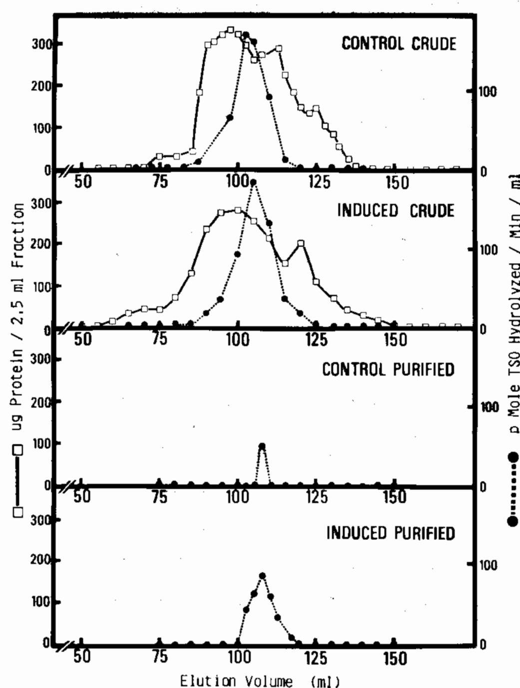

FIG. 8. Comparison of TSO hydrolysis by crude cytosol (top two panels) and affinity purified cytosolic EH (bottom two panels) from control and clofibrate-fed mice following gel permeation column chromatography on Sephacryl S-300. The R_f values (V_e/V_o) of the four cytosolic EH activities are 1.89, 1.91, 1.95, and 1.92, respectively. Comparison of R_f's with known standards indicates an approximate molecular weight of 60,000 in each case.

As demonstrated earlier (13) the MCT column can yield apparently homogeneous cytosolic EH in a single step. The bands obtained are identical whether from the cytosol of control or clofibrate-fed mice (Fig. 9). Wide and narrow range analytical isoelectric focusing indicated a single band of hydrolytic activity with identical pI's from control and clofibrate-fed animals on both wide and narrow range gels (14, 16). Similarly, the affinity purified cytosolic EHs from control and clofibrate-fed animals comigrate with each other and with catalytic activity from the cytosol (Fig. 10). Careful examination of the narrow range gels indicates a very minor band focusing at slightly higher pI than the cytosolic EH from both control and clofibrate-fed mice.

Figure 11 provides additional evidence for similarity. When the cytosolic EHs were partially purified by affinity chromatography from hepatic tissue of control and clofibrate-fed animals and used for peptide mapping under four different con-

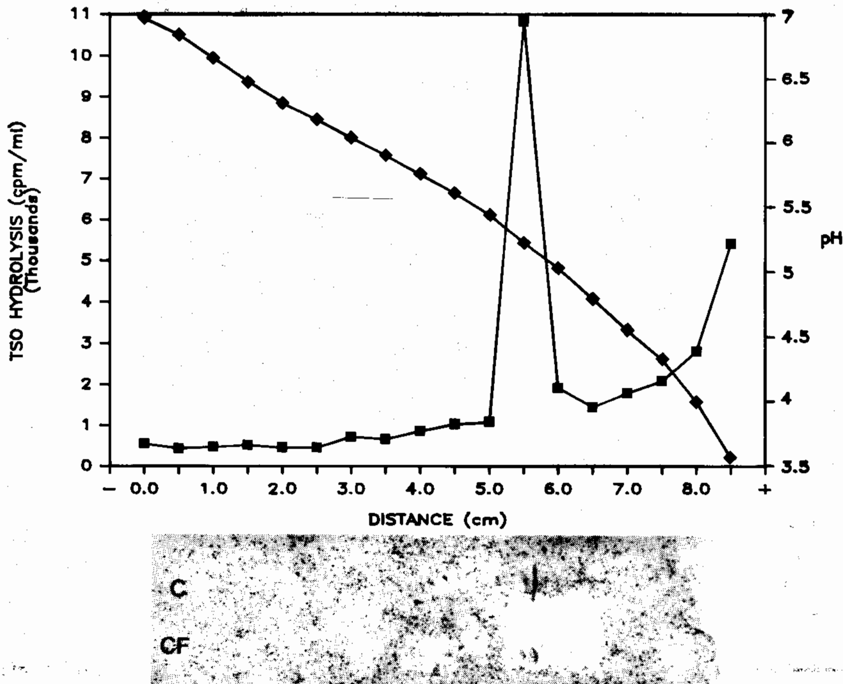

FIG. 10. Isoelectric focusing of affinity purified cytosolic EH from control (C) and clofibrate-fed (CF) mice. TSO hydrolytic activity (■) and pH (♦) of the sliced gel are shown in the upper panel while Coomassie blue stained protein following light protein loading is shown in the lower panel.

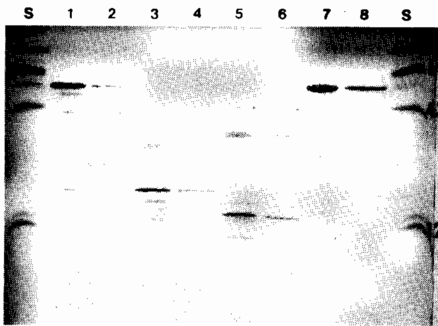

FIG. 11. SDS-PAGE analysis run under denaturing conditions of protease digests of affinity purified cytosolic EH from control and clofibrate-fed (CF) mice. Track 1, control cEH, 20 μg; Track 2, CF cEH, 30 μg; Track 3, control cEH plus 3 μg of α-chymotrypsin; Track 4, CF cytosolic EH plus 3 μg α-chymotrypsin; Track 5, control cEH plus 3 μg of V_8 protease, Track 6, CF cytosolic EH plus 3 μg of V_8 protease; Track 7, control cEH plus 3 μg of DPCC treated Trypsin; Track 8, CF cytosolic EH plus 3 μg of DPCC-treated trypsin; Track S, molecular markers, Phosphorylase *b* (92 kDa), bovine serum albumin (68 kDa), egg albumin (43 kDa), and cytochrome *c* (12 kDa).

ditions, apparently identical patterns were obtained. Some minor differences were apparent in the intensity of the bands observed following staining of SDS gels. However, in each case, including very faint bands, peptides of apparently identical R_f's were observed in the two tracks corresponding to cytosolic EH from control and clofibrate-fed mice. Although amino acid compositions for most large proteins are similar, the lack of large differences in amino acid composition between the enzyme purified by affinity chromatography from clofibrate-fed mice (Table II) and that purified by classical techniques from control animals (12) fails to contradict our hypothesis of similarity.

For Western blot analysis, preparations containing a major impurity at 30 kDa were utilized (Fig. 12). This impurity was a major component when citronellol or geraniol epoxides were used to elute mouse cytosol from the MCT Sepharose column and appeared periodically as various other

TABLE II

APPROXIMATE AMINO ACID COMPOSITION OF THE CYTOSOLIC EPOXIDE HYDROLASE FROM CLOFIBRATE-FED MICE[a]

Amino acid	nmol/ml[b]	Amino acid	nmol/ml[b]
Asx	44.4	Ile	26.1
Thr	29.4	Leu	45.2
Ser	29.6	Tyr	11.4
Glx	59.2	Phe	26.4
Pro	32.9	Lys	28.6
Gly	36.8	His	7.5
Ala	39.0	Arg	21.3
Val	28.6	Trp	NA
Met	4.5	Cys	NA

[a] Affinity purified cytosolic hydrolase was further purified by SDS-PAGE followed by electrochemical elution. Analyses were performed by A. Smith of the peptide analysis laboratory at U. C. Davis.

[b] Numbers indicated are raw analytical results divided by two to correct for a dilution factor during analysis. These data indicate an approximate molecular weight of 60 kDa for those amino acids analyzed or approximately 63 kDa if average values of Trp and Cys are included in the calculation.

elution regimes were examined. The results indicate strong reaction between the antibody raised to the cytosolic EH purified from the clofibrate-fed mice and the enzyme partially purified from each source providing further evidence for the similarities of the forms. Only a very minor reaction was observed with the 30-kDa impurity indicating that is has few antigenic determinants in common with the 60-kDa protein.

Evidence for additional epoxide hydrolases in the cytosol. The affinity column and subsequent biochemical analyses provide strong evidence that a single enzyme in the cytosol hydrates TSO; however, previous data from this laboratory indicates that other EHs are present in the cytosol. Loury *et al.* (16) and Moody *et al.* (17), both noted the presence and the slight increase of hydrolytic activity on CSO in the cytosol when mice were treated with clofibrate. This increase is anticipated since a variety of laboratories have detected the microsomal EH in the cytosol by both enzymatic and immunological assays (4). As seen in Fig. 5, the hydrolytic activity on BPO is separated clearly from the vast majority of the TSO activity by the MCT Sepharose column. Since the cytosolic EH does not detectably metabolize BPO, the small amount of cytosolic EH bleeding from the column in the loading fraction cannot account for this BPO activity. Our tentative hypothesis is that this activity is attributable to a solubilized form of the microsomal EH.

Examination of hydrolytic activities on CSO in Fig. 5 is more interesting. In the case of both control and clofibrate-fed mice, much of the activity on CSO coelutes with the TSO activity when 4-azidochalcone oxide is added to the column at B. In both cases hydrolytic activity on CSO is greater at pH 7.4 than at pH 9.0 and occurs at approximately 3 and 2% of the rates observed for TSO hydrolysis as expected for the cytosolic EH.

The loading fractions present a somewhat different situation. CSO activities are quite low in the case of control cytosols. The scales used for TSO activity in Fig. 5 make it difficult to see, but some of the CSO activity can be accounted for by a small

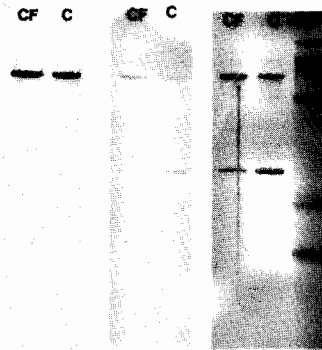

FIG. 12. Comparison of affinity purified cytosolic epoxide hydrolase by Western blot analysis from clofibrate-fed (CF) and control (C) mice. The right panel shows SDS-PAGE of 60 kDa EH and 30 kDa impurity following Coomassie blue staining. Each track contained 32 μg of protein. The center panel is SDS-PAGE after electrophoretic transfer following Coomassie blue staining. The left panel is Western blot analysis using a rabbit anticytosolic epoxide hydrolase from clofibrate-fed mice.

amount of TSO hydrolytic activity failing to bind to the column in this run. As expected for the classical cytosolic EH, hydrolysis of CSO in the loading fraction of control cytosol occurs faster at pH 7.4 than at pH 9 as it does in the affinity purified fraction from both columns.

In contrast, the relatively high rate of CSO hydrolysis in the loading fraction from the clofibrate-fed mice cannot be totally accounted for by TSO hydrolytic activity failing to bind to the column. One also can see that BPO hydrolysis is similar in both loading fractions while CSO hydrolysis is dramatically increased in the case of clofibrate-fed animals. Unlike the other three fractions, CSO hydrolysis occurs faster in the clofibrate loading fraction at pH 9 than at 7.4 also providing evidence against total involvement of the cytosolic EH. Thus, an EH activity appears to exist which clearly is distinguishable from the classical microsomal and cytosolic EHs. The hydrolytic activity demonstrated by this fraction is low, but it may be significant if better substrates for this enzyme can be found.

CONCLUSION

The MCT-based affinity procedure for the cytosolic EH has potential application to a variety of problems. Obviously, it facilitates the rapid purification of the cytosolic EH in high yield and thus the protein chemistry, immunology, and molecular biology which rely upon the ready availability of pure protein. The MCT procedure also may serve as a model for the use of stable epoxide mimics in the development of affinity procedures for other EHs of interest or pharmacologically active epoxide mimics in vertebrates.

The high yield obtained with the MCT procedure facilitates a variety of studies of EH activity including comparisons of activities in different species, organs, and subcellular fractions. Its value certainly is illustrated by this report where it facilitated testing the hypothesis that the EH activities on TSO in the cytosols of control and clofibrate-fed animals are due to the same enzyme. As one tests such an hypothesis, an obvious experimental approach is to purify the enzyme from treated and control animals. Even if the resulting pure enzymes appear to be identical by several techniques, the low yields often associated with classical purification procedures lead one to question if the proteins purified are representative of the enzyme activities observed in crude preparations. The high yields obtained with the MCT columns allow one to state with confidence that the enzyme responsible for most, if not all, of the TSO hydrolysis in mouse cytosol appears as a single band on SDS electrophoresis and by a variety of criteria is indistinguishable from the corresponding EH in clofibrate-treated animals.

An unexpected benefit of the MCT affinity column was development of clear evidence for the existence of an EH activity in the liver cytosol of clofibrate-fed mice which is distinct from the classical solubilized microsomal EH and the cytosolic EH. The essentially quantitative removal of the cytosolic EH with MCT Sepharose unmasked the low activity toward CSO present in the loading fraction. The application of MCT Sepharose to analyze cytosolic fractions from tissues of other species with and without prior exposure to xenobiotics also could prove interesting.

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