Comparison of Crude and Affinity Purified Cytosolic Epoxide Hydrolases from Hepatic Tissue of Control and Clobibrate-Fed Mice

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Received May 21, 1985, and in revised form August 6, 1985

An affinity purification procedure was developed for the cytosolic epoxide hydrolase based upon the selective binding of the enzyme to immobilized methoxytetronellyl thioli. Several elution solutions were examined, but the most successful system employed selective elution with a chalcone oxime. This affinity system allowed the purification of the cytosolic epoxide hydrolase activity from livers of both control and clobibrate-fed mice. A variety of biochemical techniques including pH dependence, substrate preferences, 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 clobibrate-fed animals. The quantitative removal of the cytosolic epoxide hydrolase acting on trans-stilbene oxide from 100,000g supernatants, allowed analysis of remaining activities acting differentially on cis-stilbene oxide and benz[a]pyrene 4,5-oxide. Such analysis indicated the existence of a novel epoxide hydrolase activity in the cytosol of mouse liver preparations.

Compounds containing the oxirane functionality (i.e., epoxides) include important biosynthetic intermediates such as isoselenic oxide and isoulotriene Λ4, environmental contaminants such as hepta- chloro epoxide, industrial intermediates like bis[1-pheno] 1,2-epoxy-1,2-epoxydiethylether and numerous plant and animal natural products. In addition, epoxides are often important inter-

mediates in the conversion of lipophilic ar-
omatic and olefinic compounds into hydrophobic 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 en-
zymes 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 en-
zymes are known as epoxide hydrolases (EC 3.2.2.3, formerly hydrases or hydra-
tases) (1-4). There now is excellent evidence for at least three epoxide hydrolases (EHs) in

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1 This work was supported, in part, by grants from NIH (ES02179-04), (R.D.H.), NSP (PCM-8011189) (G.D.P.), and the Herman Frasch Foundation (R.D.H. and G.D.P.).

2 NIEHS Research Center Development Award (ES000177). Author to whom correspondence should be addressed.


4 Abbreviations used: EH, epoxide hydrolase; TSO, trans-stilbene oxide; CST, cis-stilbene oxide; BPO, benz[a]pyrene 4,5-oxide; 30, 30-styrene oxide; NSAP, 1-nitroso-2-bromostyrene or p-nitrophenacyl.

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0003-0882/86 $3.00

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mammals; a predominantly microsomal form hydrating many xenobiotics (1-4), a microsomal enzyme system the cholesterol esters 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 chemical 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). Hammersk and Ota (14) and Lowry 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 vigorously 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 cytosol from control and clofibrate-fed mice continue to support the hypothesis that these enzymes are indistinguishable.

**EXPERIMENTAL PROCEDURES**

Chemicals.Unless noted otherwise, chemicals and solvents were from various chemical suppliers and were used without further purification. *Streptomyces aureus* VA enzyme was from Miles Laboratories (Napierville, Ill.) and all other biochemicals were from Sigma (St. Louis, Mo.). Clofibrate (ethyl 4-chlorophenoxyacetoxyisobutyrate) 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 [7]benzylcysteine reduction of deoxy chloride followed by treatment with base (20) (Fig. 1). Unlabelled benzylcysteine 4:5-oxide (BPO) was provided by Dr. Richard Armstrong (Department of Chemistry, University of Maryland). Trichloroethylene glycol 45-oxide was provided by Dr. Richard McManus (then at NIH), and trichloroethylene 34-oxides were provided by Dr. Alex Sevianov (Institute of Toxicology, University of Southern California). Unlabeled CSO was prepared by oxidation of the corresponding chlorin with trichlorophenol hypochlorite.

![Fig. 1. Structures of substrates (a-e), affinity ligand (f), and inhibitors (g-j) used in this study. a, trans-stilbene oxide; b, cis-stilbene oxide; c, benzylcysteine 45-oxide; d, trichloroethylene glycol 45-oxide; e, cytochrome P50; f, 7-ethoxycoumarin; g, 4-fluoroacetate oxide; h, 4:5-oxide; i, 4-phenylphenol; j, citronellal oxide; k, styrene oxide; l, 1,1,1-trichloropropene oxide; m, 4-nitrobenzenesulfonate.](image-url)
trichlorophenol oxide (TCP0), triis hydroxymethylaminomethane, 4-nitro-1-benzenemethophenone (NBAFP), 1,4-butanediol diglycol ether, 1-hexanol, isobutanol, were from Aldrich Chemical Company (Milwaukee, Wis.). Geranil and citronellol 61-epi and citronellol 65-epi were prepared by epoxidation of the appropriate acetates in dichloromethane with methylenephosphonic acid followed by base hydrolysis. Chalcone oxides were prepared by aldo condensation of the appropriately substituted aldehydes and acetophenones followed by conjugate addition of hydroxylamine anion as described earlier (21). Preparation of the 4 and 6-acidolactone oxides is described below (Fig. 3).

4-Acidolactone oxide. The 4-acidolactone phenoxime, mp 45-47°C, was prepared in 81% yield from treatment of 4-acidolactone with 48% HBr and NaN3 in aqueous azene (22). Condensation with benzyldine in the dark in ethanol containing 2 N NaOH for 1 h gave 64% of crude 4-acidolactone (re crystallized from ethanol), mp 93.5-96.5°C (23). Epoxidation by adding 1 N NaOH to 30% H2O2 and the chalone gave a 60% yield of re cristallized (EtOH) 4-acidolactone oxide, mp 106-107°C.

4-Acidolactone oxide. Reduction of 4-nitroalcohol oxide (21) with SnCl2-2H2O in cone HCl gave, after basic workup and recrystallization (98% EtOH), 4-acidolactone oxide in 68% yield, mp 152-154°C (24). This product was stirred with 48% HBr and NaN3 in aqueous acetone and recrystallized (EtOH) to give the 4-acidolactone in 64% yield, yellow needles, mp 153-157°C (25). The 4-acidolactone oxide, mp 80-81°C, was obtained in 81% yield from basic hydrogen peroxide in methanol. The high resolution mass spectrum of this new compound had a parent ion of m/z 260.0882, calc. for C19H18NO6, 260.0882. NMR and IR data were almost consistent with this structure.

Experimental animals. Olate-treated, male, Swiss-Webster mice (28-30 g) received 0.5% (w/w) of the drug added to ground Purina rodent chow in 5 ml/90 g corn oil for 14 days while control animals received only the diet and oil. Cytox and washed microsomes were prepared as previously described (14, 16, 17). The cytox was either used immediately or frozen at -79°C (14). One freeze-thaw cycle has been found not to cause major changes in enzyme activities.

Protein and enzyme assay. Protein levels were estimated using a previously described modification (17) of the method of Bradford (22). Data were collected and analyzed using an Atari 400 microcomputer interfaced with a Gilford Manual ELA reader.

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

The micromolar EH, at its pH optimum (ca. 8.0), rapidly hydrolyzes CIO and BPO at similar rates, while TSO metabolism by this enzyme is insignificant relative to the cytox enzyme. Thus, rates of hydrolysis of TSO and BPO were used as indicators of activity of classical cytox EH and micromolar EH, respectively, regardless of the fraction in which the activity occurred. CIO hydrolysis was corrected for the contribution by both micromolar and cytox EH in mouse liver (26).

For kinetic studies, the hydrolysis of TSO was analyzed by the spectrophotometric method of Hansa
gaus and Hammock (30) using substrate concentration
terms ranging from 1.67 x 10-4 to 1 x 10-3 M. The decrease in absorbance was monitored for at least 5
COMPARISON OF CRUDE AND PURIFIED EPOXIDE HYDROLYASE

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.

Hydrolysis of BPA at a final concentration of 5 x 10^{-6} M was monitored by a slight modification (4, 13) of the method of Jervis et al. (31). Care was taken to minimize chromoluminiscence. Hydrolysis of the 5- and 3'-halogenated epoxides previously shown to be insensitive in the cryptol of mice (Borjan and Hamscho, unpublished). Thus, a slight modification of the above procedure was used to monitor hydrolysis of these substrates using purified cryptol EHE (29, 31). A final concentration of 5 x 10^{-6} M was used for these substrates, approximating the limit of their solubility at 6 x 10^{-5} M (31).

Cytosolic glutathione (GSH) 5-transferase activity was assayed using CS0 at 5 x 10^{-5} M in pH 7.4 phosphate buffer with 5 mM GSH in a manner similar to that used for the cryptol EHE except that the reaction was terminated by the addition of hexanal. Hexanal extracts both the dye and the epoxide from the radiolabeled conjugate which remains in the aqueous phase (29). At least three replicates were run for each reported data point. In general, the activity was analyzed as described previously by this laboratory (28, 29, 29).

Inhibition of TSO hydrolysis using crude cryptol and purified cryptol EHE. For these studies the cryptol or pure protein 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 epoxides 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, 0.5 μl of ethanol containing tritiated TSO (5 x 10^{-5} M final ethanol) 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.

For initial studies on the suitability of various inhibitors to be used in affinity chromatography, the cryptol fractions from control and chlorinated-treat mice were diluted in 76 mM sodium phosphate buffer (pH 7.4). Four milliliters of the extract containing 10 μg of the appropriate inhibitor diluted in ethanol (to give 80-96% inhibition) were preincubated for 15 min at 37°C. The samples were added to 4-μl-diameter, washed, Spectraprep (Los Angeles, Calif) dialysis sacks, and identical but separately dialyzed against 800 ml of sodium phosphate buffer at 0°C. At various times during dialysis, three 100-μl aliquots were removed and assayed for cytotoxicic activity using 5 x 10^{-5} M TSO. Buffers were changed at 2, 6, 10, and 22 h. Alternatively, 0.3 ml of a 4% solution of sodium metabisulfite added, diluted with 1 ml of buffer (pH 7.4) was added to 1.5 ml purified cytotoxicic EHE and the fractions dialyzed as above.

Preparation of affinity columns. The affinity matrix was prepared essentially as described previously (13). To Sepharose CL-6B (wash with 10 vol of distilled water) was added 28 ml of 1 N NaOH containing 57 mg of NaH2PO4 and 5.7 ml of 1.4-butanediol (dibutryl) ether. The slurry was stirred at room temperature for 2 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 methanol-filtered to remove most liquid. Analysis of the free epoxy functionality with sodium bisulfite routinely yielded 5-15 μmol/g Sepharose (wet wt) (30).

The affinity ligand was prepared by organomercury-demethylation of crotonylated, conversion to the thiol form by the Mitsuobu procedure (30), and reduction of the free thio-pentamethylthiopentyl thiol (MCT) with LAdH (27) (Fig. 1). Approximately a 2-fold excess of MCT in ethanol was added to the epoxy-activated matrix in 1/3 methanol-water containing 0.01 N NaHCO3. After stirring for 24 h, the gel was exhaustively washed and stored at 4°C in absolute ethanol containing 0.2% butylated hydroxyanisole. Before loading, the gel was exhaustively washed with 76 mM sodium phosphate buffer (pH 7.4), 0.1 mM sodium EDTA, 0.1 mM dithiobisulfite (DTT) which was subsequently used for loading, washing, and eluting with several additions.

Affinity purification of cytotoxic EHE. A variety of procedures were used to optimize the affinity purification of the cytotoxic EHE using the MCT gel. Optimization procedures were carried out at 2 ± 0.2°C using a 1.5 to 2.5 ml bed volume of 8-9 ml i.d. Wheaton reflux condensers serving as coolant-jacketed mini-columns. Cytotoxicic was diluted to 2% (original liver w/v) in the EUTA, DTT, phosphate buffer described above and passed through the MCT-Sepharose column of 0.2-0.3 ml/min using a peristaltic pump. Pull-out a column wash using a volume of buffer at least equal to the volume of column loaded on the column several column elution regimens were evaluated by adding various reagents to the wash buffer at the above flow rate. Fractions were assayed as described above for TSO, CS0, and BPA hydrolysis as well as GSH conjugation.

Preparative studies were carried out on 2-4°C using the modified procedure above. After washing with 76.2 ml of buffer (8 ml bed vol) with 700-1000 ml of buffer at 0.5-0.9 ml/min, it was immediately loaded with hepatic cryptol (12.2% liver w/v) from control or experimentally treated mice (1800-2800 total) or elaborated (500-500 ml) treated mice at 0.5-0.6 ml/min. The column was subsequently washed with approximately 1 liter of buffer at 0.8-0.9 ml/min, then cytotoxicic EHE was then eluted with 45-50 ml of a saturated solution of 4-nitrophenol.
oxide in buffer. Following protein analysis, samples containing chalone oxide were dialyzed by one of several procedures described below and all samples analyzed as above. Alternatively, the column was eluted with 0.5 x 10^-4 M 4-thiouracil and using the above procedure.

Gel permeation. A Sephacryl S-300 column (1.6 x 85 cm, Pharmacia) was poured and equilibrated with 50 mM sodium phosphate buffer, pH 7.5, 0.1 M EDTA, and 0.02% sodium azide then calibrated with Blue Dextran, bovine serum albumin, ovalbumin, myoglobin, and cytochrome c. Cytosolic ES samples (30% cytosol from control mice (43 mg protein/ml), 10% cytosol from colchicine-fed mice (68 mg protein/0.5 ml), affinity purified cytosolic cysteine ES (16 mg protein/ml), affinity purified colchicine-induced cysteine ES (26 mg 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 1 ml/h. Fractions (3.5 ml) collected were assayed for 3H tyrosine activity and protein content.

Gel electrophoresis. Deoxo-sodium polysulfide-induced gel electrophoresis (SDDS-PAGE) was carried out as described by Lasenby (38) with a Hoefer Vertical slab gel system (SE 400, Hoefer Scientific Instruments, Molecular markers included phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (45,000), aspartic transamin inhibitor (35,000), 20,000), and cytochrome c (12,000). Gels were stained with 0.1% Coomassie brilliant blue in ethanol/water/ acetic acid (30/115/5) 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 protein digestion 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 1107 Multigel System). Wide range gels used pH 3.5 to 9.5 or pH 5 to 8 Ampholines (LKB), while narrow range gels were run with pH 4 to 6 or 4.3 to 4.8 polyacrylamide gels. Evans blue (pH 4.6) was used as the tracking dye. After electrophoresis, lanes containing ES were cut into 0.5 cm segments and incubated overnight at 4°C in 1 ml of distilled water. The next day, pH 7.8 histidine activity of the supernatant was measured. Gels were also fixed with 1% acetic acid and 11.5% trichloroacetic acid, soaked in methanol, 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 buffer 0.25% SDS, 6 M Tris-HCl, pH 6.8, 1% DTT, 1 mM EDTA, and 3% mercaptoethanol. After cooling, the samples were loaded into the wells of a 15% SDS-PAGE gel, and 3 µg of respective protein 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. Proteins used included DPC-treated cystein, chymotrypsinogen, and S. aureus V8 protease. For non-denatured protein, 30 µg of the enzyme (25 ng pH phosphate buffer, pH 7.8) was digested with 1/100 (w/w) chymotrypsin, added for 2 h at 37°C. At the end of the incubation, the protein was blended in the sample buffer to inactivate the protease and then run on a 15% SDS-PAGE gel. Amido acid analyses were performed on the cytosolic ES by A. Smith of the University of California, Davis, peptide laboratory following electrophoretic elution of the affinity purified material from SDS-PAGE (40).

RESULTS AND DISCUSSION

Development of electrolysis systems for MCT Sepharose. It was demonstrated previously that MCT Sepharose is capable of retaining the cysteolic ES activity for TSO present in a large volume of mouse cytosol while most of the GSH transferase activity passed through the column (13). Since most of the substrates and inhibitors of the cytosolic ES, including MCT, are lipophilic, the hypothesis was developed that the enzy-

mecolumn interaction largely was hydrophobic. Thus, a step gradient of the nonionic detergent, Lubrol-PX, was used. The cytosolic ES activity eluted between 0.5 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 non-specific binding of cysteolic proteins to the MCT gel, a more specific elution was required to obtain homogeneous enzyme. However, these data indicate that moderate concentrations of ethylate 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 cysteolic EH.

pH profiles were very similar to cEH activity in normal and cultured 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 cysteolic 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 cysteolic 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 cysteolic EH although some additional GSH transferase activity did elute from the column. Subsequent elution with 4-aza-deoxochalcone oxide as described later indicate that the cysteolic EH activity was not desaturated 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 cysteolic 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 cysteolic EH (45, 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 88% of the loaded cysteolic EH activity at concentrations far below those used with Lubrol indicates biospecific elution. This column gave a 30-fold purification with recovery of 80% of the cysteolic 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 36,000 also are eluted with these terpenoid epoxides in addition to the 60,000 M, band for the cysteolic EH as shown by SDS-PAGE. This conclusion 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-aza-deoxochalcone oxide (see below) and Lubrol-PX failed to elute additional cysteolic EH, although the Lubrol did elute additional protein.

A number of inhibitors were examined as possible ligands for the elution of the cysteolic 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 NBT 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 cysteolic 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 as an essential sulfhydryl group in the enzyme and possibly could be considered a suicide substrate. The inhibition of the cysteolic
Fig. 3. Affinity purification of cytosolic EH from control mouse liver cytosol. Fig. 3A shows elution with a chalcone oxide. Elution of Protein (C) and dEH (D) from MCT Sepharose. A, 70 mM sodium phosphate buffer, pH 7.4; B, pH step gradient, 5 ml each of pH 7.4, 6.5, 6.0, 5.5, 5.0; C, 4-chalcone oxide, 5 ml each of $1 \times 10^{-9}$ M, $1 \times 10^{-8}$ M, $1 \times 10^{-7}$ M; D, Lubrol-PX, 0.05% (5 ml), 0.1% (5 ml). This column gave a 92-fold purification of the cytosolic EH in 27% yield with a specific activity of 356 nmol/min/mg protein following dialysis to remove the chalone oxide. Fig. 3B shows elution with a terpene oxide. Elution of protein (C) and cytosolic EH (D) from MCT Sepharose: A, 70 mM sodium phosphate buffer, pH 7.4; B, Lubrol-PX, 0.1%; C, citrusfurfuryl epoxide, 10 ml each of $1 \times 10^{-8}$ M, $1 \times 10^{-9}$ M, and $1 \times 10^{-10}$ M; D, 4-chalcone oxide, $5 \times 10^{-8}$ 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, longer courses of inhibition indicate that maximal inhibition is obtained within minutes, which in 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) at $6 \times 10^{-9}$ M, $8 \times 10^{-9}$ 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 E3H by selected compounds: ethanol control (○), 4-fluorobenzene oxide (1 x 10⁻⁸ M, △), 4-phenylbenzene oxide (1 x 10⁻⁸ M, ▽), and stigasone oxide (2.5 x 10⁻⁸ M, □). Methods as described in text.
ative (Fig. 1, 4-hydroxyphenyl naphthalene,
entoxanthyl manganese, \(10^{-3}\) M was successfu
The enzyme appeared homogeneous on
SDS-PAGE, but complete removal of the
inhibitor required 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 studi
ies, total activity from cytosolic EH inhibi
ted with slightly less potent compounds

![Graph 1](image1)

![Graph 2](image2)

**Fig. 5.** Elution of peptide hydrolyse activities from paired MCT gels using cytosolic from control (left two panels) and elodehase-fed mice (right two panels). In both cases the upper graph shows the hydrolytic activity on TSG at pH 7.4 (A) and BPO at pH 9 (B) while the lower two graphs indicate the hydrolysis of CSD at pH 7.4 (C) and pH 9.0 (D). Note that the scale for hydrolysis of TSG is different from other substrates. In each case 60 ml of 2% (original wet wt/m vol) gel was freshly prepared from control or elodehase-fed mice was pumpe into equivalent 2.5-mL beds of MCT-Septase. The columns were eluted with the following regime: A, wash with buffer; B, elute with 1 mM 4-nitroacetone oxide C, wash with buffer.
such as the 4-fluoro, 4-azido, or 4-azidochalcone oxides ($I_m$'s $2 \times 10^{-4}$, $1.6 \times 10^{-4}$, and $4.8 \times 10^{-4}$ 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 $I_m$, and its removal can be monitored spectrophotometrically (Fig. 3, 5, Table I). The azidochalcone oxides were prepared as photofluority 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 eluent from the MCT column, cytosolic EH activity can be detected with TSO even in the presence of chalcone oxides. This property is of obvious

![Diagram](attachment:image.png)
Table I

<table>
<thead>
<tr>
<th>Protein (mg)</th>
<th>TSO Hydrolysis pH 7.4</th>
<th>CBS Hydrolysis pH 7.4</th>
<th>BPO Hydrolysis pH 9.0</th>
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<td></td>
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<td>Clotbrate</td>
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* Activities loaded and eluted are expressed in units defined as nmol/min while specific activities are nmol/min/mg protein. Control (single experiment) and clotbrate (average of two experiments) cytosols are described in Methods.

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

benefit in the optimization of columns 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 homogenous 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 homogenous preparations from control and clotbrate-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 cytosol of control and clotbrate-fed mice is due entirely to the same, single isolated enzyme. Similarities in substrate selectivity, inhibition on analytical iso-electric focusing, and apparently identical Rf's on SDS electrophoresis provided a strong indication that the cytosolic EH from liver (and in some cases kidney) of control and clotbrate-treated mice were identical[14, 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 clotbrate-fed animals behave similarly on the column and upon subsequent SDS-PAGE. The enzyme activities elute with similar structures and concentrations of ligands (Fig. 6) 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 equal 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 clotbrate cytosols. Similar stabilities of the affinity purified cytosolic EHs from livers of control and clotbrate-fed mice during dialysis provide additional evidence for identity.

It has been noted previously that clotbrate 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 8.5%, respectively). Similar trends can be seen as one compares eluted TSO activity with CSO activity run at both 7.4 and 9.9 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. Hammond and Hasagawa (27) pointed out that TESO is hydrated over 16 times faster in mouse cytosol than in 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 22 times faster than TSO, and other lipid epoxides hydrated still faster, one can anticipate velocities of the cytosolic EH of well over 90,000 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 medium 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 cyclohexene 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 was 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, 210 μg and clofibrate 79, 148, 156, and 294 μg protein added) and the affinity purified cytosolic EH (0.68 ± 0.04 μg) indicating that velocities were linearly dependent on protein concentration. Kinetic constants (Vmax is mmol/min/mg protein and Km, app to atmol) were determined as described by Wilkinson (33) and are as follows for control cytosol (8.4 ± 0.28, 4.75 ± 0.51), clofibrate cytosol (15.8 ± 0.4, 4.13 ± 0.41), purified control (96 ± 7, 4.61 ± 1.18), and purified clofibrate (44± 2, 3.71 ± 0.97).
In addition to initial rates, the kinetics of hydrolysis of TSO appear similar. Of special note, the $k_{cat}$'s are essentially identical in cytisol and affinity purified preparations from both control and clofibrate-fed mice. The $V_{max}$ was increased in crude cytisol 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 cytisol and affinity purified preparations is shown. The close similarity of the lines from the two preparations is surprising for styrene oxide and NBP 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 NBP 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 cytisol 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-Trichloroethylene oxide is a potent inhibitor of the microsomal EH (1) but weak inhibitor of the cytosolic EH (2). Inhibition appears also to be reversed as the inhibitor is hydrated. 4-Phenyldichloro oxide is the most potent inhibitor reported for the cytosolic EH possibly acting as a transition state mimic. The inset in Fig. 7 indicating that the potency of 4-PCO varies with the concentration of the enzyme is an indication of near stoichiometric inhibition which often is true of transition state inhibitors. Inhibition slowly decreases when experiments were performed in triplicate and the values shown are the mean of two separate experiments using cytisol from different mice.
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 optimum 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 TS0 hydrolytic activity are indistinguishable by gel permeation on Sepharose 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 TS0 eluting just after bovine serum albumin. These data indicate that the monomeric (50 kDa SDS, 55 ± 5 kDa gel permeation) as well as the dimeric forms (48 of the enzyme retain catalytic activity.

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, 15). 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 pH 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 EII from control (C) and clofibrate-fed (CF) mice. TSG hydrolytic activity (A) and pH (B) of the stained 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 EII from control and clofibrate-fed (CF) mice. Track 1, control EII 20 μg; Track 2, CF EII, 30 μg; Track 3, control EII plus 5 μg of α-chymotrypsin; Track 4, CF cytosolic EII plus 5 μg α-chymotrypsin; Track 5, control EII plus 5 μg of V$_2$ protease; Track 6, CF cytosolic EII plus 5 μg of V$_2$ protease; Track 7, control EII plus 5 μg of DPC comb-tryspin; Track 8, CF cytosolic EII plus 5 μg of DPC-comb-tryspin; Track 9, molecular markers, Phosphorylase b (92 KDa), bovine serum albumin (66 KDa), egg albumin (45 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/s were observed in the two tracks corresponding to cytosolic EII 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 HCT Sepharose column and appeared periodically as various other
TABLE II
APPROXIMATE AMINO ACID COMPOSITION OF
THE CYTOSOLIC EPoxide HYDROLASE FROM
CLOFibrate-FED MICE

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>mmol/mg</th>
<th>Amino acid</th>
<th>mmol/mg</th>
</tr>
</thead>
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<tr>
<td>Asx</td>
<td>44.4</td>
<td>Ile</td>
<td>26.1</td>
</tr>
<tr>
<td>Thr</td>
<td>29.4</td>
<td>Leu</td>
<td>45.2</td>
</tr>
<tr>
<td>Ser</td>
<td>29.6</td>
<td>Tyr</td>
<td>11.4</td>
</tr>
<tr>
<td>Gln</td>
<td>59.2</td>
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<tr>
<td>Met</td>
<td>4.5</td>
<td>Cys</td>
<td>NA</td>
</tr>
</tbody>
</table>

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

** Numbers indicated are raw analytical results di-
  vided by two to correct for a dilution factor during
  analysis. These data indicate an approximate molec-
  ular weight of 60 kDa for these amino acids analyzed
  or approximately 63 kDa if average values of Trp and
  Cys are included in the calculation.

The elution regimes were examined. The results
indicate strong reaction between the an-
tibody raised to the cytosolic EH purified
from the clofibrate-fed mice and the en-
gy ne partially purified from each source
providing further evidence for the similar-
lities of the forms. Only a very minor re-
an action was observed with the 30-kDa im-
purity indicating that is has few antigenic
determinants in common with the 60-kDa
proteins.

Evidence for additional epoxide hydrolase
species 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 hy-
drocobal activity on (SO) in the cytosol when
mice were treated with clofibrate. This in-
crease is anticipated since a variety of lab-
oratories have detected the microsomal EH
in the cytosol by both enzymatic and im-
 munological 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 SCT Sepharose column.
Since the cytosolic EH does not detectably
metabolize BPO, the small amount of cy-
tosolic 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 sol-
ubilised 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,
most of the activity on CSO coelutes with
the TSO activity when 4-azidoacene 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 ap-
proximately 3 and 2% of the rates observed
for TSO hydrolysis as expected for the cy-
tosolic EH.

The loading fractions present a some-
what 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

** Bild von der Abbildung 12.**

** Bild von der Abbildung 13.**
amount of TSO hydrolytic activity failing to bind to the column in this run. As expected for the classical cytosolic EH, hydrolysis of C50 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 C50 hydrolysis in the loading fraction from the elobrate-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 C50 hydrolysis is dramatically increased in the case of elobrate-fed animals. Unlike the other three fractions, C50 hydrolysis occurs faster in the elobrate 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 latter 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 purifications 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 cytosol of control and elobrate-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 elobrate-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 elobrate-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 unmasks the low activity toward C50 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.

REFERENCES
