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DISTRIBUTION AND NATURE OF EPOXIDE HYDROLASE ACTIVITY IN SUBCELLULAR ORGANELLES OF MOUSE LIVER

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Abstract—Mouse liver light and heavy mitochondrial fractions contain significant epoxide hydrolase activity in addition to that present in the cytosol and microsomes. As the mitochondrial fraction itself contains a number of subfractions, experiments were designed to determine the localization of the epoxide hydrolase activity in these subfractions. Subcellular fractions were prepared using livers from 6- to 8-week-old Swiss–Webster male mice. Using *trans*-stilbene oxide (TSO) as substrate, the highest activity was localized in the cytosolic fraction, followed by the light mitochondrial fraction. Subfractionation of the light mitochondrial fraction by isopycnic sucrose density gradient resulted in the separation of mitochondria from peroxisomes as monitored by marker enzymes. The separation of these two subcellular organelles was also confirmed by the electron microscopic studies. Distribution of TSO-hydrolase activity in the sucrose density gradient fractions closely resembled the activity distribution of the peroxisomal markers catalase and urate oxidase, but significant activity was also found in mitochondria. Treatment of mice with clofibrate selectively induced TSO-hydrolase in the cytosol without affecting this enzyme activity in the peroxisomal fraction. There was no difference in the distribution pattern of TSO-hydrolase and marker enzymes in sucrose density gradients of mitochondrial fractions from clofibrate-treated and control mice. The epoxide hydrolase activity in the peroxisomes is immunologically similar to, and also has the same molecular weight as, the cytosolic epoxide hydrolase.

As a number of epoxides are highly reactive and have toxic, mutagenic, and carcinogenic properties, the ability of epoxide hydrolases (EC 4.2.1.63) to convert epoxides to dihydrodiols has been recognized as an important metabolic step in the detoxification of these compounds [1, 2]. Epoxide hydrolase activity has been reported to occur in the microsomal [3], nuclear [4], mitochondrial [5, 6], and cytosolic [7] fractions of mammalian liver. The epoxide hydrolases in the microsomal and cytosolic fractions differ in their selectivity toward various substrates [8], their pH optima for a single substrate [8], their distribution in various animal species [9], and immunological characteristics [10]. However, the epoxide hydrolase activities in cytosolic and mitochondrial fractions are similar with respect to their substrate selectivity [6], its response to various inhibitors [6], and in its immunological properties [11]. However, evidence is still needed to confirm that the epoxide hydrolase activity is due to the same protein in these two fractions.

Clofibrate, the hypolipidemic drug and peroxisome proliferator, causes an increase in the cytosolic and microsomal epoxide hydrolase activity in mouse liver when administered in the diet [12–14]. Its effect on epoxide hydrolase activity in mitochondrial fraction, however, remains to be established. Significant amounts of epoxide hydrolase activity have been reported previously in the heavy and light mitochondrial fractions of mouse liver [6]. Although in those experiments maximum epoxide hydrolase activity was detected in mitochondria which were separated from lysosomes, no attempt was made to separate other subcellular particles such as peroxisomes, and the data were interpreted on the basis of results obtained from mitochondrial fractions containing peroxisomes. Subsequent studies have showed that epoxide hydrolase activity is also localized in the peroxisomes of mice liver [15].

For a proper consideration of the role of epoxide hydrolase in liver metabolism, reliable knowledge as to its subcellular location is required. The present study was therefore designed to gain a better understanding of the epoxide hydrolase in the mitochondrial fraction of livers of control and clofibrate-treated mice.

MATERIALS AND METHODS

Chemicals. MOPS [3-(*N*-morpholine)propanesulfonic acid], Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA), and mannitol were obtained from the Sigma Chemical Co., St. Louis, MO. Other reagents were of analytical grade or the highest purity commercially available. Clofibrate [ethyl-α-(4-chlorophenoxy)-α-methylpropionate] was synthesized by BF_3 catalyzed transesterification of clofibric acid [16] and was >95% pure. [^{3}H]*trans*- and [^{3}H]*cis*-stilbene oxides (TSO

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and CSO respectively) were synthesized as previously reported [17] and had a >97% geometrical purity and a >99% radiochemical purity.

Treatment of animals. Male Swiss–Webster mice (6- to 8-weeks-old, 20–25 g) were allowed free access to clofibrate or control diet and tap water up to the time they were killed. Clofibrate diet was made by incorporating clofibrate dissolved in corn oil into ground rodent chow. The final concentration of clofibrate and corn oil in the diet was 0.5% (w/w) and 10% (v/w) respectively. Control animals received a similar diet containing corn oil alone. Experimental animals were maintained on their diet for 10 consecutive days.

Preparation of subcellular fractions. Animals were killed, without prior fasting, by cervical dislocation. Their livers were dissected, perfused with ice-cold 0.25 M sucrose, and weighed. All subsequent procedures were performed at 0–4°. Pooled livers from control and clofibrate-treated mice were homogenized in 0.25 M sucrose containing 0.3 M mannitol, 10 mM Hepes, 1 mM EGTA, pH 7.2, and 0.1% ethanol to prevent the formation of inactive catalase. Differential sedimentation and density gradient fractionation were performed in a Sorvall SA 600 angle rotor and Beckman VTi50 vertical rotor respectively.

The liver homogenates (10%, w/v) were centrifuged at 600 *g* for 5 min to separate cell debris and nuclei. The pellet was rehomogenized in about the same quantity of the buffer and centrifuged at 600 *g* for 10 min. After repeating this operation a second time, the nuclear sediment, then almost free of gross debris, was redispersed by means of homogenizer and made up to a final volume equal to four times the weight of the tissue processed yielding a 1:4 nuclear fraction. The 600 *g* supernatant fractions were made up to volume to form 1:10 cytoplasm extract and centrifuged at 6000 *g* for 10 min to obtain a pellet and a supernatant fraction. The 6000 *g* pellet was resuspended in approximately the same volume of 0.25 M sucrose solution containing 2 mM MOPS, 5 mM EGTA and 0.1% ethanol, pH 7.2, and centrifuged at 6000 *g* for 10 min to sediment heavy mitochondria. The process was repeated once, and finally the pellet was resuspended in the medium to obtain a 1:8 heavy mitochondrial fraction. The 6000 *g* supernatant fraction was then centrifuged at 12,000 *g* for 15 min to obtain the light mitochondria and the postmitochondrial fraction. The pellet was washed two times with 0.25 M sucrose containing 2 mM MOPS, 5 mM EGTA and 0.1% ethanol, pH 7.2, and finally resuspended in the medium to give a 1:8 light mitochondrial fraction. The postmitochondrial supernatant fraction was centrifuged at 105,000 *g* for 60 min to obtain the microsomal pellet and the cytosol fraction. The microsomal pellet was resuspended in the original homogenizing medium and washed two times. Finally, the microsomes were resuspended to give a 1:4 microsomal fraction. The cytosolic fraction was used without further purification.

Density-gradient fractionation. Continuous sucrose gradients were used to separate mitochondria and peroxisomes [18]. Approximately 2 ml of heavy mitochondrial fraction (35–45 mg protein) and 2 ml of light mitochondrial fraction (8–15 mg protein) from control and clofibrate-treated mice were layered on top of a 42–51% (w/w) continuous linear sucrose gradient (gradient volume 26 ml) containing 2 mM MOPS, 1 mM EGTA and 0.1% ethanol, pH 7.2. A cushion of 4 ml of 60% (w/w) sucrose solution and an overlay of 3 ml of 100 mM sucrose solution were incorporated. The remaining top part of the tubes was filled with mineral oil and the tubes were sealed.

Centrifugation was performed at 4° in a Beckman VTi50 rotor at 52,000 *g* for 60 min with slow acceleration and deceleration. After centrifugation, twenty-two 1.5-ml fractions were collected from the top of the gradient by means of a density gradient fractionator, and they were assayed for marker enzyme and epoxide hydrolase activities.

Marker enzyme assays. Marker enzymes were used to follow the separation of subcellular fractions. The activities of the peroxisomal enzyme markers, catalase (EC 1.11.1.6) and urate oxidase (EC 1.7.3.3), were assayed as described [19], while succinate dehydrogenase (SDH) activity (EC 1.3.99.1) was monitored by following the reduction of $K_3Fe(CN)_6$ to $K_4Fe(CN)_6$ [20]. Citrate synthase (EC 4.1.3.7), an enzyme marker of the mitochondrial matrix, was assayed as reported earlier [21]. Monoamine oxidase (MAO) activity (EC 1.4.3.4) was determined by monitoring the formation of benzaldehyde [22], while glucose-6-phosphatase activity (EC 3.1.3.4) was assayed by following the liberation of inorganic phosphate from glucose-6-phosphate [23]. Acid phosphatase (EC 3.1.3.2), a marker for lysosomal activity, was assayed using *p*-nitrophenyl phosphate as substrate [19]. Lactate dehydrogenase (EC 1.1.1.27), a marker for cytosol, was assayed at 340 nm following published procedures [19].

Epoxide hydrolase assay. Epoxide hydrolase activity in different subcellular fractions was monitored by partition assay methodology [17]. Briefly, epoxide hydrolase activity in the cytosolic, and heavy and light mitochondrial fractions was monitored with TSO (5×10^{-5} M final concentration) in 0.1 M sodium phosphate buffer, pH 6.8, while microsomal epoxide hydrolase activity was monitored with CSO (5×10^{-5} M final concentration) in 0.1 M Tris–HCl buffer, pH 9.0. After 10-min incubations, the reaction was stopped by addition of *n*-dodecane, and the mixture was vortexed and centrifuged. The aqueous phase containing the product diol was quantitated by liquid scintillation counting.

Subcellular fractions and density gradient fractions of heavy and light mitochondrial fractions were diluted to a suitable concentration with appropriate buffer immediately before assay. Protein concentrations were determined according to Bradford [24].

Electron microscopic studies. Separate mitochondrial and peroxisomal rich fractions obtained after density gradient centrifugation of the light mitochondrial fraction were processed for electron microscopic studies. Fractions 7 and 14 which were rich in mitochondria and peroxisomes, respectively, were diluted with 0.25 M sucrose containing 2 mM MOPS, 1 mM EGTA and 0.1% ethanol, pH 7.2. The mitochondrial preparation was centrifuged in an Eppendorf centrifuge for 30 sec, while the peroxisomal

preparation was centrifuged at 30,000 *g* for 30 min in a Beckman ultracentrifuge to sediment the organelles. The supernatant fractions were discarded, and the pellets were taken up in 2% (w/v) agar and handled with care throughout the procedure. The pellets were placed in 35% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, for 3 hr at room temperature and washed three times with the same buffer. After 12 hr, the pellets were postfixed in 1% buffered osmium tetroxide for 2 hr and rinsed three times in buffer, followed by careful dehydration in a graded series of acetone and propylene oxide. Finally, the propylene oxide was removed and replaced first with a 50% mixture of Epon 812 and propylene oxide, and then with pure Epon 812 which was allowed to polymerize for 48 hr at 60°. Silver-gold sections were cut with a diamond knife, stained with 1% uranyl acetate in 100 mM maleate buffer, pH 7.2, saturated methanolic uranyl acetate and Reynold's lead citrate, and examined under a Hitachi 100 S electron microscope at 75 kV.

Purification of cytosolic epoxide hydrolase and preparation of antibody. Mouse liver cytosolic epoxide hydrolase was prepared according to the methods previously published [25], and antibodies to this protein were raised in adult New Zealand white rabbits as described [11]. Control serum was obtained from nonimmunized rabbits. The serum from immunized rabbits gave a single precipitin line in Ouchterlony double diffusion analysis when tested against the purified enzyme [11].

Double diffusion analysis. Double diffusion analyses were performed in 1% agarose gels containing 100 mM Tris–HCl buffer, 50 mM sodium azide, 1% polyethylene glycol 8000 and 0.9% NaCl layered on glass slides. Undiluted serum (15 μl) was added to the center well and 20 μl of solubilized mitochondrial, microsomal or peroxisomal fractions, and crude cytosol from control and clofibrate-treated mice were loaded in the outer wells. The plates were incubated in a moist chamber at 37° for 18 hr before visualization.

Immunoprecipitation of epoxide hydrolase activity. Light mitochondrial fraction from control and clofibrate-treated animals, and isolated mitochondrial and peroxisomal fractions obtained after density gradient centrifugation from control animals were used for immunoprecipitation experiments. Mitochondrial and peroxisomal fractions were solubilized by the addition of Lubrol PX (final concentration 1%). After solubilization, the subcellular fractions were diluted with 0.1 M phosphate buffer, pH 6.8, to give a suitable protein concentration before use. Solubilized fractions (100 μl) were incubated with various amounts of immune serum (0–20 μl) in a final volume of 1 ml. Nonimmune serum was added, if necessary, to all tubes so that the final amount of serum (immune and nonimmune) was 20 μl in each tube. After overnight incubation at 4°, 100 μl of IgGsorb (resuspended mixture) was added to each tube and incubated for 30 min at 4°. The tubes then were centrifuged at 2000 *g* for 10 min. The supernatant fraction was removed, and the precipitate was resuspended in 0.1 M phosphate buffer, pH 6.8, with vigorous vortexing. Both the supernatant and the precipitate were analyzed for epoxide hydrolase activity. Control experiments contained IgGsorb and serum, but no enzyme protein. Supernatant and precipitated fractions from these incubations were used as enzyme blanks in the enzyme activity assays.

RESULTS

Clofibrate treatment did not cause any significant change in the body weights of mice. The average liver weight was, however, significantly higher (24.6%) than that of the untreated control group. The relative liver size (liver weight expressed as a percentage of body weight) increased significantly (16.1%) after clofibrate treatment. In addition, clofibrate treatment led to a 15.5% increase in total hepatic protein content.

Subcellular distribution of marker enzymes. The mitochondrial marker enzymes SDH, citrate synthase and MAO were found to be localized mainly in the heavy mitochondrial fraction (Fig. 1). The peroxisomal marker enzymes, catalase and urate oxidase, were distributed predominantly in the heavy and light mitochondrial fractions. Although the specific activities of these peroxisomal enzymes were highest in light mitochondrial fraction, total enzyme activities were higher in the heavy mitochondrial fraction. Glucose-6-phosphatase, a microsomal marker, was recovered mainly in the microsomal fraction. The specific activity of this enzyme was, however, higher in the light mitochondrial fraction probably due to microsomal contamination. However, this microsomal contamination of mitochondria was not crucial because TSO, which was used as a substrate to monitor epoxide hydrolase activity in the mitochondrial fraction, is a poor substrate for the microsomal epoxide hydrolase [17]. Lactate dehydrogenase was predominantly located in the 105,000 *g* supernatant fraction. However, the distribution of acid phosphatase was irregular with the highest levels recorded in heavy and light mitochondrial fractions.

Subcellular distribution of epoxide hydrolases. TSO-hydrolase activity was localized mainly in the cytosolic fraction followed by the heavy and light mitochondrial fractions (Fig. 1). Of the total TSO-hydrolase activity recovered (80.9%) in various subcellular fractions, 3.3, 15.5, 6.7, 0.9 and 73.2% of the activity was present in cell nuclei, heavy mitochondria, light mitochondria, microsomes and cytosol respectively. Of the CSO-hydrolase activity recovered (78.9%), 5.7, 10.8, 11.2, 60.4 and 11.9% was recovered in the respective fractions. No marked differences in the distribution pattern of TSO-hydrolase in comparison with catalase were noted. With CSO as substrate, the subcellular distribution of epoxide hydrolase was very similar to that observed with glucose-6-phosphatase.

Distribution of protein in subcellular fractions. Total protein recovered in the different liver subcellular fractions was 76%. This recovery is similar to the recovery of both TSO- and CSO-hydrolases. Thus, it is likely that the loss in protein content and epoxide hydrolases probably occurred during the repeated washings of the pellets.

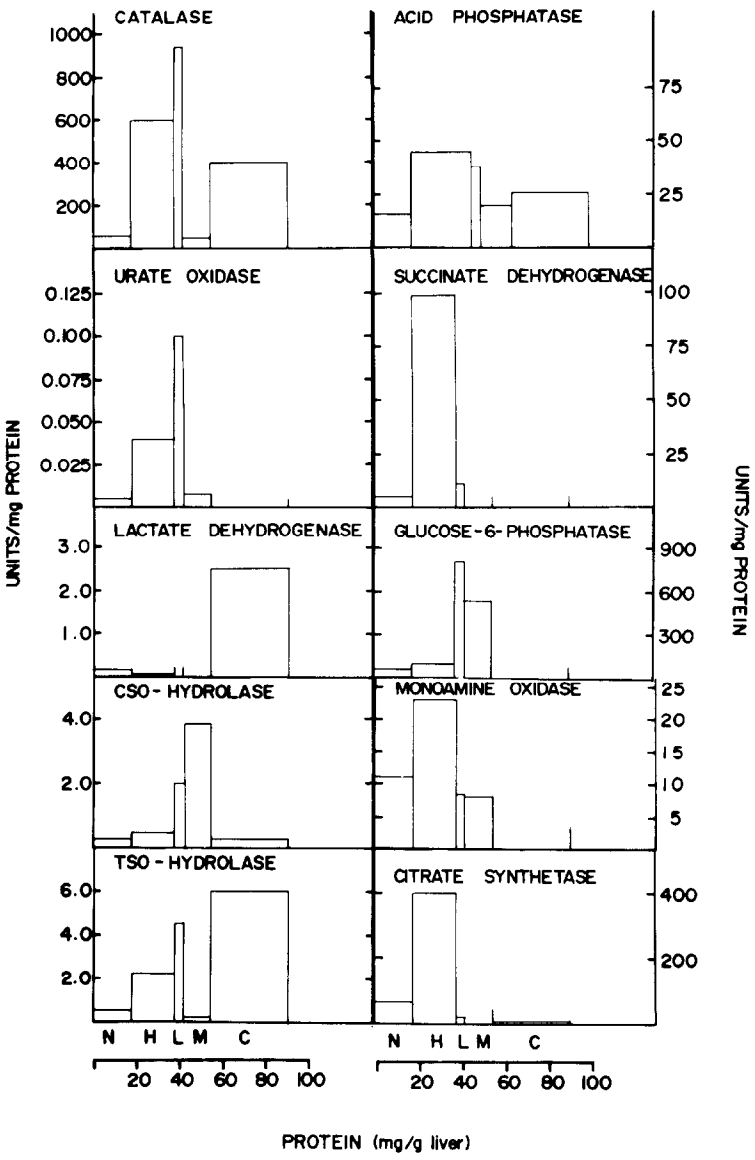

Fig. 1. Distribution of TSO-hydrolase, CSO-hydrolase and marker enzymes in subcellular fractions of mouse liver. Fractions which do not show histograms have no detectable enzyme activity. Abbreviations: N, nuclei; HM, heavy mitochondria; LM, light mitochondria; M, microsomes; and C, cytosol.

Subcellular distribution of marker enzymes and epoxide hydrolases obtained by differential sedimentation in mice treated with clofibrate. Clofibrate administration to mice did not cause any significant change in the distribution pattern of the marker enzymes and epoxide hydrolases. In comparison to controls, however, elevated levels in the specific activities of catalase and urate oxidases (123 and 173% respectively) were noted in the light mitochondrial fraction from clofibrate-treated mice. The specific activities of mitochondrial, microsomal, lysosomal and cytosolic marker enzymes were not altered in the various isolated subcellular fractions after clofibrate treatment. In contrast, there was a 2.1- and 1.8-fold increase in TSO- and CSO-hydrolase activities in the cytosolic and microsomal fractions, respectively, in clofibrate-treated animals. However, the specific activity of TSO hydrolase in the light mitochondrial fraction of clofibrate-treated animals was reduced to approximately half of that in the light mitochondrial fraction of untreated animals (Fig. 2). There was no significant change observed, however, in the distribution of TSO- and CSO-hydrolase activities in the other subcellular fractions between control and clofibrate-treated animals.

Total activities of epoxide hydrolases and marker enzymes in subcellular fractions from control and clofibrate-treated mice. In general, clofibrate treatment caused an increase in the total activities of the enzymes examined, even though the specific activities remained the same (Table 1). Total TSO-hydrolase activity in the cytosol and CSO-hydrolase

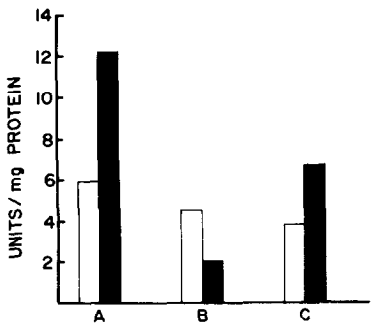

Fig. 2. Effect of clofibrate treatment on hydrolysis of *trans*-stilbene oxide (TSO) and *cis*-stilbene oxide (CSO) in subcellular fractions. (A) TSO-hydrolase in cytosolic fraction, (B) TSO-hydrolase in mitochondrial fraction, and (C) CSO-hydrolase in microsomal fraction. Key: (□) control, and (■) clofibrate. Each bar represents the mean of two experiments done in triplicate.

activity in the microsomal fraction were induced 3- and 3.5-fold, respectively, following clofibrate treatment, whereas there was no significant change observed in TSO-hydrolase enzyme activity in the light and heavy mitochondrial fractions. However, CSO-hydrolase activity was increased in all fractions except in the cytosol.

Equilibrium density centrifugation. To further maximize the separation of mitochondria from peroxisomes, the light mitochondrial fraction was subjected to isopycnic centrifugation in a linear sucrose gradient (Fig. 3). The mitochondria which were retained on the upper part of the gradient constituted the major part of the protein, whereas peroxisomes which constituted the minor part of the protein were banded at greater densities than mitchondria. Although it was not possible to obtain peroxisomes completely uncontaminated with mitochondria, some of the fractions were relatively pure as confirmed by electron microscopy (Fig. 4). Subfractionation of the heavy mitochondrial fraction on sucrose gradient showed a distribution profile essentially similar to that noted with light mitochondrial fraction. There were also no apparent differences observed in the distribution pattern of heavy and light subcellular mitochondrial fractions from clofibrate-treated mice when these preparations were subfractionated on sucrose density gradient. However, the total activities of TSO hydrolase, CSO-hydrolase and other marker enzymes were increased when compared to preparations obtained from untreated mice (Table 1). The specific activities of marker enzymes, except that of catalase and urate oxidase, however, did not change significantly (data not shown).

The distribution of TSO-hydrolase activity based

Table 1. Epoxide hydrolase and marker enzyme activities in subcellular fractions of livers from control and clofibrate-treated mice

	Enzyme activity* (units/g liver)				
	Fractions				
Enzyme	Nuclei	Heavy mitochondria	Light mitochondria	Microsomes	Cytosol
Control					
MAO	211	476	37.8	116	ND
Citrate synthase	2,570	8,320	114	ND	329
SDH	91	2,010	260	ND	ND
Catalase	1,160	2,360	4,120	750	14,800
Urate oxidase	0.09	0.89	0.44	0.09	ND
Acid phosphatase	291	931	167	272	951
Glucose-6-phosphatase	1,250	2,240	3,500	7,250	ND
LDH	2.9	0.8	ND	0.5	92
CSO-hydrolase	4.9	9.3	9.6	51.7	10.2
TSO-hydrolase	10	46	19.8	2.7	217
Clofibrate					
MAO	112	612	97	36	23
Citrate synthase	2,990	12,000	645	156	548
SDH	318	2,530	ND	ND	ND
Catalase	2,460	16,500	10,600	3,160	32,700
Urate oxidase	0.1	1.7	1.6	0.8	ND
Acid phosphatase	402	1,550	378	339	1,736
Glucose-6-phosphatase	2,990	5,990	4,000	15,100	1,578
LDH	2.7	2.4	0.7	0.8	183
CSO-hydrolase	15.3	28.0	20.3	176	8.8
TSO-hydrolase	17.8	57.3	17.8	8.9	683

Units: MAO, nmoles benzaldehyde formed/min; citrate synthase, urate oxidase and LDH, 0.01 O.D. change/min; SDH, 0.001 O.D. change/min; catalase, 0.05 O.D. change/min; acid phosphatase, nmoles *p*-nitrophenol formed/min; glucose-6-phosphatase, nmoles P_i formed/min; and CSO hydrolase and TSO hydrolase, nmoles diol formed/min.

* Data are presented as the mean of two experiments done in triplicate. ND = not detectable.

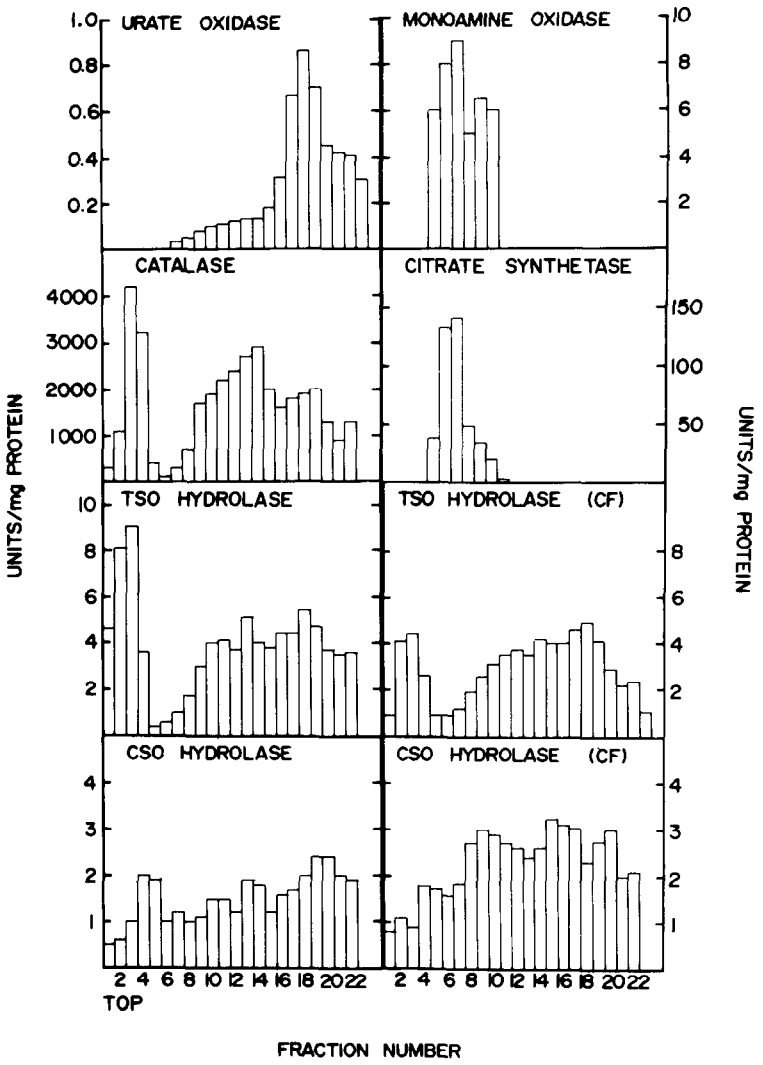

Fig. 3. Localization of TSO-hydrolase in light mitochondrial fraction of mouse liver by isopycnic subfractionation. Fractions which do not show bars had no detectable enzyme activity. CF = clofibrate. Recovery of the protein (9.5 mg) applied to the sucrose gradient was 100%.

on its specific activity showed a profile similar to that of peroxisomal marker enzymes catalase and, to a lesser extent, urate oxidase (Fig. 3). Similarly, CSO-hydrolase activity showed a distribution pattern very similar to the microsomal marker enzyme, glucose-6-phosphatase, which was recovered in most of the gradient fractions. All the fractions were also apparently contaminated with the lysosomal enzyme, acid phosphatase. However, when the fractions were analyzed for total activity instead of specific activity, significant TSO-hydrolase activity was present in mitochondrial rich fractions. For example, in density gradient fractions of the heavy mitochondrial fraction, 50% of TSO-hydrolase activity was recovered in fractions 5–8 (mitochondrial rich), even though these fractions showed low TSO-hydrolase specific activity. Similar results were obtained with the light mitochondrial fraction. TSO-hydrolase activity in fractions 1–4 was apparently due to lysis of mitochondria and/or peroxisomes.

Ouchterlony double diffusion analysis. Single immunoprecipitin lines were obtained with solubilized heavy and light mitochondrial fractions, and cytosolic fractions from control and clofibrate-treated mice when analyzed with rabbit antibody to mouse liver cytosolic epoxide hydrolase (Fig. 5A). Similarly, a single immunoprecipitin line was observed with the peroxisomal fraction obtained from isopycnic centrifugation (Fig. 5B). Under similar conditions a faint line was observed with mitochondrial fraction even though the total protein concentration applied was approximately double of that applied from peroxisomal fraction. No immunoprecipitin line was observed with solubilized mouse liver microsomal fraction.

Immunoprecipitation of epoxide hydrolase activity. Antibodies raised against purified mouse liver cytosolic epoxide hydrolase immunoprecipitated TSO-hydrolyzing activity from the light mitochondrial fraction of control and clofibrate-treated mice (Fig.

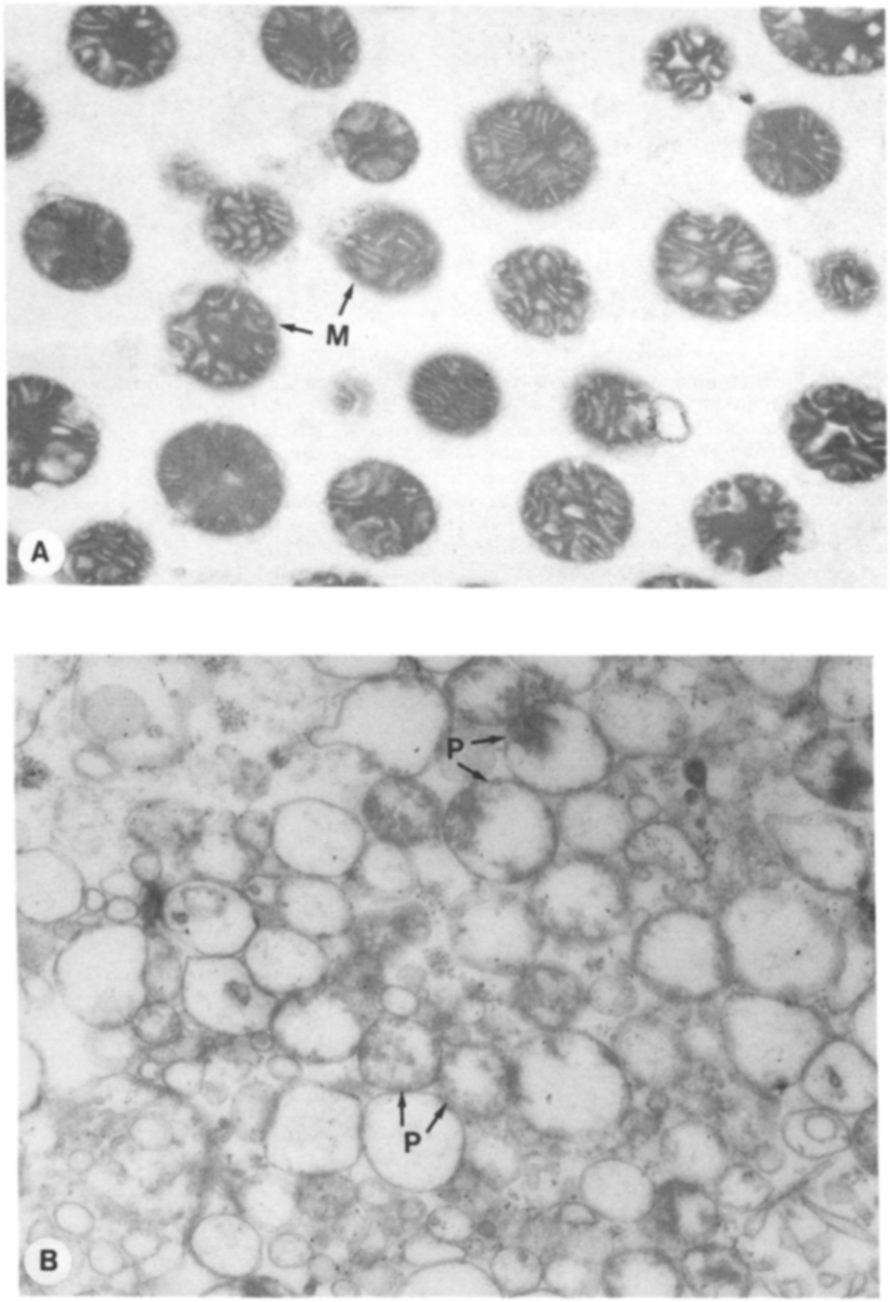

Fig. 4. Electron micrographs of the pellets prepared from isolated mitochondria and peroxisome rich fractions obtained after density gradient centrifugation. (A) M = mitochondria, magnification, 30,000; (B) P = peroxisomes, magnification, 36,000.

6). TSO-hydrolase from purified mitochondrial and peroxisomal fractions obtained after sucrose density gradient centrifugation was similarly immunoprecipitable with the antibody (Fig. 7). The TSO-hydrolase activity in the purified peroxisomal fraction required substantially less antibody for immunoprecipitation than the TSO-hydrolase activity in the purified mitochondrial fraction.

Antibody obtained from unimmunized rabbits did not immunoprecipitate any TSO-hydrolase activity from the light and heavy mitochondrial fractions, purified mitochondria and peroxisomes, and the cytosol. No detectable level of TSO-hydrolase activity was found in IgGsorb. Also, IgGsorb did not precipitate any TSO-hydrolase activity in the absence of antibody.

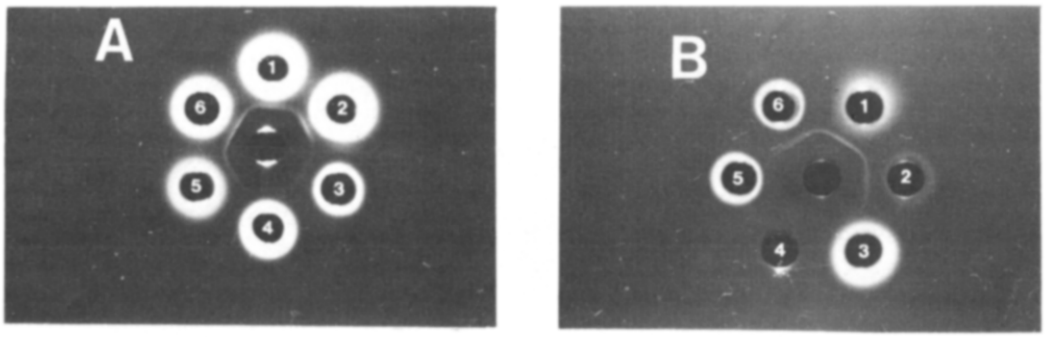

Fig. 5. Ouchterlony double diffusion analysis of epoxide hydrolase in subcellular fractions of livers from control and clofibrate-treated mice. The center wells contained 15 μl of rabbit anti-mouse liver cytosolic epoxide hydrolase. The outer wells contained the following amounts of protein. (A) Wells 1 and 2 contained 406 and 400 μg of solubilized heavy mitochondria from control and clofibrate-treated mice respectively. Wells 3 and 4 contained 104 and 167 μg of solubilized light mitochondria from control and clofibrate-treated mice respectively. Wells 5 and 6 contained 84 and 108 μg of crude cytosol from control and clofibrate-treated mice respectively. (B) Well 1 contained 84 μg of crude cytosol. Wells 2 and 3 contained 106 and 186 μg of solubilized peroxisomal and mitochondrial fractions, respectively, obtained after density gradient centrifugation. Wells 5 and 6 contained 148 and 104 μg of solubilized microsomal and light mitochondrial fractions respectively.

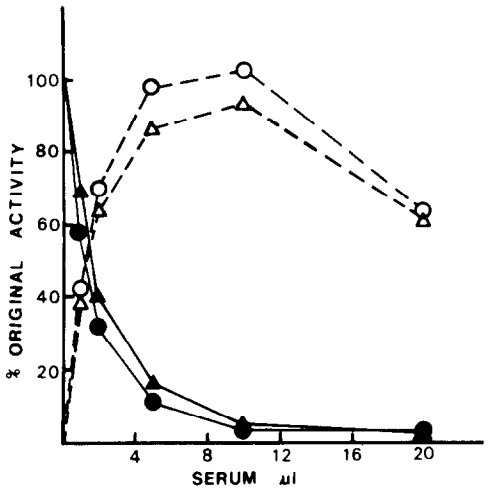

Fig. 6. Immunoprecipitation of TSO-hydrolase in crude mitochondrial fraction from control and clofibrate-treated mice with antibody to cytosolic epoxide hydrolase. Epoxide hydrolase activity was measured with TSO as substrate. Symbols are as follows: In control mice, (●—●) mitochondria and antibody, EH activity in the supernatant; and (○---○) mitochondria and antibody, EH activity in the immunoprecipitate. In clofibrate-treated mice, (▲—▲) mitochondria and antibody, EH activity in the supernatant; and (△---△) mitochondria and antibody, EH activity in the immunoprecipitate. One hundred percent activity of enzyme refers to the amount of activity present after carrying out the immunoprecipitation incubation in the presence of 20 μl of nonimmune serum.

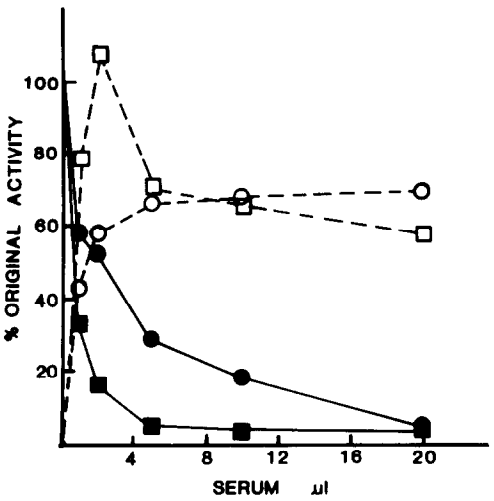

Fig. 7. Immunoprecipitation of TSO-hydrolase in isolated mitochondrial and peroxisomal fractions obtained after density gradient centrifugation from control mice. Symbols are as follows: (●—●) mitochondria and antibody, EH activity in the supernatant; (○---○) mitochondria and antibody, EH activity in the immunoprecipitate; (■—■) peroxisomes and antibody, EH activity in the supernatant; and (□---□) peroxisomes and antibody, EH activity in the immunoprecipitate.

DISCUSSION

The subfractionation and isopycnic centrifugation procedures utilized in this study gave a distribution pattern of marker enzymes very similar to those reported earlier [26, 27]. The TSO-hydrolase was localized predominantly in the cytosol, with substantial activity in the light and heavy mitochondrial fractions. The specific activity of TSO-hydrolase was lower in heavy mitochondrial fraction, but total activity, as expected, was higher when compared with that of light mitochondrial fraction. These results are very similar to studies performed previously with a different substrate [6].

The pattern of TSO-hydrolase activity recovered in the gradient following isopycnic subfractionation

closely parallelled the distribution profile of peroxisomal markers catalase and urate oxidase. This distribution pattern raises the possibility of the peroxisomal origin of TSO hydrolase as suggested [15]. However, catalase has been shown to exist as five main heteromorphs with differential tissue and subcellular distribution, with the acidic heteromorph being characteristic of peroxisomes, whereas more basic forms occur in the extraparticulate cytoplasm [28, 29]. Significant TSO-hydrolase activity was present in the mitochondrial rich fractions (fractions 5–8), but the specific activity was much lower in these fractions than that in the peroxisomal rich fractions. While it is possible that the mitochondrial fraction could be contaminated with peroxisomes, the low level of contamination as detected by electron microscopy, and assuming catalase is not present in the mitochondria, indicates that a significant portion of the TSO-hydrolase activity recovered in the mitochondrial fraction may reside in the mitochondria. It is unlikely that this mitochondrial TSO-hydrolase activity is due to the lysosomal contamination because earlier studies with Triton WR-1339-treated mice [6] showed that lysosomes have little TSO-hydrolase-like activity. Nevertheless, this study shows that the major portion of TSO-hydrolase activity in the light mitochondrial fraction is in peroxisomes. The epoxide hydrolase activity in the peroxisomes, as previously suggested for the mitochondria [6], appears to be localized in the peroxisomal matrix as the enzyme activity was detectable in lysed peroxisomes.

The results of Ouchterlony double diffusion analysis are consistent with those reported earlier [11] and suggest the presence of a protein, in crude and purified mitochondrial fractions, and the peroxisomal fraction, which is antigenically similar to cytosolic epoxide hydrolase. The peroxisomal and cytosolic epoxide hydrolases also have a similar molecular weight.* No immunoprecipitin line was observed with microsomes, confirming earlier studies that show the mouse cytosolic and microsomal epoxide hydrolases to be distinct proteins [11, 12, 25]. These results also show that the peroxisomal epoxide hydrolase is antigenically different from the microsomal epoxide hydrolase.

Antibodies raised against purified cytosolic epoxide hydrolase were able to immunoprecipitate TSO-hydrolyzing activity from the mitochondrial and peroxisomal fractions. However, significantly less antibody was required to immunoprecipitate epoxide hydrolase activity in the peroxisomal fraction probably due to the lower level of total epoxide hydrolase present in the peroxisomal fraction, even though the specific activity is higher. Additionally, it is possible that the presence of high protein concentration in the mitochondrial fraction does interfere with the ability of the antibody to immunoprecipitate the epoxide hydrolase. The cause for inhibition of TSO-hydrolyzing activity in the peroxisomes at high antibody titers is not clear, although it is possible that inhibitory antibodies are present in the serum.

Administration of clofibrate, diethylhexylphthalate, 2-ethylhexanol and nafenopin to mice has been shown previously to induce cytosolic TSO-hydrolase [12–15, 30]. As is evident from the results presented here, clofibrate treatment did not cause an increase in the specific and total activities of TSO-hydrolase in the mitochondrial and peroxisomal fractions. This difference in the responses of epoxide hydrolases catalyzing TSO hydrolysis in cytosol, and mitochondria and peroxisomes suggests that the epoxide hydrolases in these fractions may be differentially regulated.

Although epoxide hydrolases play an important role in the metabolism of epoxides, their physiological importance is unclear. However, recent evidence suggests that the epoxide hydrolases may play a significant role in the degradation of endogenous epoxides [31, 32]. The epoxide hydrolases could also be involved in the degradation of epoxides formed as a result of auto-oxidation [33, 34]. The toxic and potentially carcinogenic cholesterol epoxide is apparently generated in cell membranes during lipid peroxidation by interaction of cholesterol and hydroperoxides [34]. Similarly, under physiological conditions, finite levels of epoxides of arachidonic acid and other unsaturated fatty acids may be present in the cell as a result of lipid peroxidation, auto-oxidation or epoxidation. An inability to readily metabolize these epoxides could render various cellular components at risk to the epoxides.

The significance of epoxide hydrolase activity in the peroxisomal fraction is not known. Since peroxisomal membranes contain large amounts of polyunsaturated fatty acids and are potential sites of lipid peroxidation, auto-oxidation and epoxidation resulting in cellular damage, it might be possible that the presence of epoxide hydrolase in peroxisomes acts as a biological protector system in metabolizing these endogenous lipid epoxides formed during various deteriorative reactions. However, it is unlikely that the peroxisomal epoxide hydrolase is important in the metabolism of cholesterol epoxide, as purified cytosolic epoxide hydrolase, which is immunologically similar and has similar substrate specificity as the epoxide hydrolase in the peroxisomal fraction, does not significantly metabolize both α and β cholesterol epoxide (S. S. Gill, unpublished observation). The peroxisomal epoxide hydrolase is, however, capable of metabolizing fatty acid epoxides formed during auto-oxidation [6].

The mechanism of induction of cytosolic epoxide hydrolase by peroxisomal proliferators is not understood. Earlier studies have shown that nafenopin binds to a cytosolic protein in rats, resulting in an increase in the number of peroxisomes and associated peroxisomal enzymes [35]. Whether induction of cytosolic epoxide hydrolase is under the control of this hypothesized cytosolic receptor which binds to clofibrate requires further investigation.

The significance of induction of cytosolic epoxide hydrolase by peroxisomal proliferators is unclear. The hypolipidemic peroxisome proliferators, e.g. clofibrate, have been considered as a novel class of chemical carcinogens [36]. The carcinogenicity of these compounds, unlike the majority of other chemical carcinogens, is apparently associated with the proliferation of peroxisomes and activation of the peroxisomal hydrogen peroxide generating oxidases,

* S. S. Gill and S. Kaur, unpublished observations.

which result in a sustained increase in intracellular hydrogen peroxide thereby causing DNA damage [37, 38]. Thus, the cytosolic epoxide hydrolase could be simultaneously induced to metabolize potentially toxic lipid epoxides formed in peroxidation.

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