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EPOXIDE HYDROLASE ACTIVITY IN THE MITOCHONDRIAL AND SUBMITOCHONDRIAL FRACTIONS OF MOUSE LIVER

SARJEET S. GILL* and BRUCE D. HAMMOCK

Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, CA 92521, U.S.A.
and
Departments of Entomology and Environmental Toxicology, University of California, Davis, CA 95616, U.S.A.

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Abstract—Distribution of epoxide hydrolase activity in subcellular fractions of livers from male Swiss–Webster mice and Sprague–Dawley rats was monitored with *trans*-β-ethylstyrene oxide, *trans*-stilbene oxide and benzo[*a*]pyrene 4,5-oxide following differential centrifugation. With the former two substrates the highest activity was encountered in the cytosolic fraction; however, significant activity was found in the mitochondrial fraction. These fractions hydrated benzo[*a*]pyrene 4,5-oxide very slowly, and the major benzo[*a*]pyrene 4,5-oxide hydrolyzing activity was recovered in the microsomal fraction. Using Triton WR-1339-treated mice, it was shown that *trans*-β-ethylstyrene oxide hydrolyzing activity was predominantly localized in the mitochondria rather than in lysosomes and peroxisomes. Subsequent separation of the mitochondrial fraction into submitochondrial components by swelling, shrinking, and sonication, followed by sucrose density gradient centrifugation, showed that most of the epoxide hydrolyzing activity was present in the matrix and intermembrane space fraction. Significant activity was also present in the outer and inner membrane fractions. However, epoxide hydrolyzing activity in these fractions was reduced if either increased sonication times were used or the fractions were washed, indicating possible contamination of these fractions by the matrix and intermembrane space enzyme(s). The epoxide hydrolase activity in the mitochondrial and cytosolic fractions in mice appeared similar with regard to inhibition, molecular weight, and substrate selectivity.

Epoxidized compounds are an important group of xenobiotics which may either be present in our environment or form metabolically *in vivo* from olefinic or aromatic compounds. A number of these epoxides are potentially toxic, mutagenic and/or carcinogenic [1]. Such epoxides can undergo metabolic conjugation with glutathione (GSH), a reaction sometimes catalyzed by glutathione *S*-transferases (GSH transferases); be reduced enzymatically to the corresponding olefin; or be hydrolyzed to diols by epoxide hydrolases [2–5]. Of these metabolic routes, the greatest attention has been focused on epoxide hydrolases.

The epoxide hydrolases are ubiquitous in nature, occurring in all vertebrate species tested [6] and in a variety of other organisms. These epoxide hydrolases have also been detected in almost all mammalian tissues tested [6, 7]. Subcellular distribution studies initially indicated that styrene oxide hydrolase activity in such mammalian tissues occurred predominantly in the endoplasmic reticulum [8, 9]. Subsequent studies, however, have shown that epoxide hydrolase activity is fairly widespread in the liver cells if different compounds are utilized as substrates. For instance, a higher level of epoxide hydrolase activity is observed in the 100,000 *g* supernatant fraction than in any other subfraction if juvenile hormone mimics or epoxidized fatty acids are used as substrates [10–14], and epoxide hydrolase activity also occurs in the nuclear membrane of mammalian liver cells [15]. The nuclear epoxide hydrolase activity monitored with styrene oxide and benzo[*a*] pyrene 4,5-oxide appears to be due to the same enzyme as that present in the endoplasmic reticulum [16].

In routine subcellular distribution studies on epoxide hydrolase activity in mammalian liver, significant activity was encountered in the mitochondrial fraction [13, 14]. This study verifies the occurrence of epoxide hydrolase activities in mammalian liver mitochondria using *trans*-β-ethylstyrene oxide and other epoxides as substrates.

MATERIALS AND METHODS

Chemicals. 1-[4′-Ethyl-^{14}C-phenoxy]-3,7-dimethyl-6,7-epoxy-2*E*-octene (ethyl epoxide, 0.63 GBq†/mmole, > 96 per cent *E*) was obtained from the Stauffer Chemical Co., Mountain View, CA, U.S.A., and purified to >99 per cent as described [11, 17]. Methyl *cis*-9,10-epoxystarate was synthesized from [1-^{14}C]-oleic acid (1.1 GBq/mmole,

* Author to whom all correspondence should be addressed: Dr. Sarjeet S. Gill, Department of Entomology, University of California, Davis, CA 95616, U.S.A.

† One becquerel (Bq) equals one disintegration per second (International System of Units).

ICN Pharmaceuticals, Irvine, CA) as described [13]. *trans*-β-Ethylstyrene oxide (1.9 GBq/mmole) was synthesized and purified by high performance liquid chromatography (h.p.l.c.) as reported earlier [18]; *trans*- and *cis*-stilbene oxides (2.1 GBq/mmole) were synthesized by base treatment of the corresponding chlorohydrin obtained by reduction of desyl chloride by [^{3}H]sodium borohydride and purified by thin-layer chromatography (t.l.c., silica gel GF_{254}) using ether–hexane (10:1) as the solvent system. The products, identified by their u.v. spectra, were found to be >99 per cent chemically pure and > 97 per cent isomerically pure when cochromatographed with authentic standards in a number of solvent systems. [^{3}H]-Benzo[*a*]pyrene 4,5-oxide (37 MBq/mmole) was supplied by Professor F. Oesch (University of Mainz, Mainz, West Germany). *p*-Chlorophenoxyepoxypropane, allylbenzene oxide, and other compounds were either obtained commercially or synthesized in this laboratory by phenoxydation of the corresponding olefin with *m*-chloroperoxybenzoic acid. All other chemicals used were of analytical grade or the best grade commercially available.

Enzyme preparation. Male mice (Swiss–Webster, 30–40 *g*, 9 to 12-weeks-old) and rats (Sprague–Dawley, 200–280 *g*) obtained from Simonsen Laboratories, Gilroy, CA, were killed by cervical dislocation and by decapitation, respectively. The livers were removed immediately and rinsed in ice-cold 0.25 M sucrose. Non-hepatic tissue was discarded, and the livers were weighed and then homogenized in 0.25 M sucrose–Tris–HCl (10 mM, pH 7.4) buffer using a loose fitting Potter–Elvehjem homogenizer to give a 10% (w/v) homogenate. The homogenate was centrifuged at 600 *g* for 10 min. The precipitate was rehomogenized and recentrifuged at the same speed to give the cell nuclei and debris fraction. The two supernatant fractions were centrifuged at 6,500 *g* for 10 min, giving the heavy mitochondrial fraction. This mitochondrial fraction was resuspended and repelleted at the same speed. The process was repeated once more. The 6,500 *g* supernatant fraction was subsequently centrifuged at 12,000 *g* for 10 min to give a light mitochondrial fraction which was washed once. The 12,000 *g* supernatant fraction was subsequently centrifuged at 100,000 *g* for 60 min to give the microsomal pellet and the cytosolic supernatant fraction. The microsomal pellet was washed once. Cell nuclei and debris, heavy and light mitochondria, and microsomes were resuspended in the same buffer.

The procedure of Sottocassa *et al.* [19] was used to separate submitochondrial fractions. A portion of the heavy mitochondrial pellet was resuspended in 10 mM Tris–HCl (pH 7.5) buffer to give a 40% (w/v) homogenate based on initial homogenization. After 5 min, a one-third volume of 1.8 M sucrose containing 2 mM adenosine-triphosphate and 2 mM $MgSO_4$ was added. After an additional 5 min, the mixture was sonicated at 4–5 amp for 30–60 sec with a Branson Sonifier. The sonicated mixture was then layered on top of a 1.18 M sucrose solution and centrifuged in a Beckman SW 41 rotor for 3 hr at 100,000 *g*. Five distinct phases were obtained: a fluffy layer on top, a clear yellowish layer consisting of the matrix and intermembrane space, the outer mitochondrial membrane layer, an intermediate 1.18 M sucrose layer, and the inner mitochondrial membrane pellet on the bottom. The individual layers were removed and the pellet was resuspended in 0.25 M sucrose–Tris–HCl (10 mM, pH 7.4) buffer.

The procedure of Leighton *et al.* [20] was followed to separate lysosomes from mitochondria. Triton WR-1339 was administered to mice i.p. in 0.9% (w/v) NaCl at a dose of 850 mg/kg 3.5 days before the mice were killed. Mice were starved for 16 hr before killing; livers were removed and then homogenized as indicated earlier and the various subcellular fractions were obtained. The 6,500 *g* pellet was washed twice and resuspended in 45% (w/v) sucrose (1 ml/g of liver). Two ml of this suspension was layered on top of 2 ml of 60% sucrose. Subsequently, 5 ml of 34.5% and 2 ml of 14.3% sucrose were layered on top. The tube was then centrifuged at 80,000 *g* for 2 hr in a SW 41 rotor. Two distinct fractions were obtained. The top fraction lying between 14.3% and 34.5% sucrose was gently siphoned off from the top, while the bottom layer lying above 60% sucrose was removed by piercing the cellulose nitrate tube from the bottom. This bottom layer had separated into upper and lower portions which were collected individually.

The various subcellular and submitochondrial fractions were diluted with buffer to give appropriate protein concentrations before use. Protein concentration was determined by the method of Lowry *et al.* [21] using bovine serum albumin, fraction V, as the standard.

Marker enzyme assays. Marker enzymes were used to follow the separation of subcellular and submitochondrial fractions. The inner mitochondrial membrane marker enzyme, cytochrome oxidase (EC 1.9.3.1), was assayed by following the oxidation of sodium dithionite-reduced cytochrome *c* at 550 nm [20]. NADPH–cytochrome *c* reductase (EC 1.6.2.4), an endoplasmic reticulum marker, was assayed at 550 nm in 50 mM phosphate buffer (pH 7.5) with 0.3 mM KCN according to the method of Sottocassa *et al.* [19]. Cytochrome P-450, similarly an endoplasmic reticulum marker, was estimated by the CO-difference spectra of sodium dithionite-reduced preparations following the procedure of Omura and Sato [22]. Acid phosphatase (EC 3.1.3.2), a marker for lysosomal activity, was assayed using *p*-nitrophenyl phosphate as substrate according to Bergmeyer [23] in the presence of 0.3% Triton X-100. Enzyme assays were performed after fractions were incubated for 5 min in 50 mM sodium citrate buffer, pH 4.8. Urate oxidase (EC 1.7.3.3), a peroxisomal enzyme, was measured at 292 nm in the presence of 0.1% Triton X-100 according to the method of Kalckar [24]. Lactate dehydrogenase (EC 1.1.1.27), a marker for the cytosol, was assayed at 340 nm following published procedures [23]. Citrate synthase (EC 4.1.3.7), to follow mitochondrial matrix, was assayed by the procedure of Shepherd and Garland [25]. Adenylate kinase (EC 2.7.4.3), to monitor mitochondrial intermembrane space, was followed according to published procedures [23].

Epoxide hydrolase assays. Epoxide hydrolase activity (EC 3.3.2.3) was monitored using a variety

of epoxidized substrates. *trans*-β-Ethylstyrene oxide hydration was followed by an assay procedure based on partitioning of unreacted epoxide from the enzyme incubation into isooctane, with the diol product being retained in the aqueous phase [18]. A similar procedure, with minor modifications, was employed in assays utilizing *trans*- or *cis*-stilbene oxides as substrates. Ethyl epoxide and methyl *cis*-epoxystearate hydration was monitored following the procedures of Mumby and Hammock [26] and Gill and Hammock [13], respectively. Benzo[*a*]pyrene 4,5-oxide hydration was followed by t.l.c. according to the method of Jerina *et al.* [27]. *p*-Chlorophenoxyepoxypropane, allylbenzene oxide, and styrene oxide hydration were assayed by gas–liquid chromatography (g.l.c.) using a flame-ionization detector (2.5% OV-101 on Gas Chrom Q 100–200 mesh, 1.5 mm × 1.2 m glass column). Product diols were detected as their *n*-butyl boronic diesters, with appropriate internal standards. Unless otherwise indicated, the following concentrations of epoxides and incubation times were used: *trans*-β-ethylstyrene oxide (5×10^{-4} M, 10 min), *trans*- and *cis*-stilbene oxides, allylbenzene oxide, styrene oxide, and benzo[*a*]pyrene 4,5-oxide (5×10^{-5} M, 10 min), ethyl epoxide (2×10^{-5} M, 10 min), methyl *cis*-epoxystearate (2.2×10^{-5} M, 10 min), and *p*-chlorophenoxyepoxypropane (5×10^{-5} M, 30 min). These substrate concentrations were below their respective solubility limits. Enzyme incubations involving radiometric assays were run in 50–150 μl, whereas g.l.c. assays were run in 1 ml of enzyme preparation. In all cases, the protein concentration used was such that the appearance of diol was linear with incubation time (0–30 min). Incubation conditions were used that approached substrate saturation, and the reactions were linearly dependent upon nondenatured protein concentrations (1–20 μg/incubation).

Molecular weight estimation. Mitochondrial epoxide hydrolase molecular weight was estimated using a Sephacryl S-200 column (86 × 2.5 cm, i.d.) eluted with sodium phosphate buffer, pH 7.4, I = 0.2 M. Proteins of known molecular weight, including the cytosolic epoxide hydrolase, were used as standards as reported previously [14].

Effect of inhibitors of the cytosolic epoxide hydrolase on the mitochondrial epoxide hydrolase. Known inhibitors of the cytosolic epoxide hydrolase were assayed at concentrations greater than the I_{50} for the cytosolic epoxide hydrolase. Inhibitors were added in 1 μl EtOH to 50 μl of mitochondrial matrix and intermembrane space fraction and incubated for 10 min at 37° prior to the addition of *trans*-β-ethylstyrene oxide as substrate. After 10 min, diol formation was assayed as described above.

RESULTS

Subcellular distribution of marker enzymes and purity of fractions in normal mice. Of the total protein present in the initial homogenate of normal Swiss–Webster male mouse liver, 86 per cent was recovered in the individual subcellular fractions. A significant portion of the protein loss probably occurred during the process of washing pellets. These washes were not quantified for protein content or enzyme activity. Distribution of marker enzymes in the subcellular fractions is given in Table 1. NADPH–cytochrome *c* reductase, a marker enzyme for the endoplasmic reticulum, was present in greatest amounts in the microsomal fraction. Significant levels of the enzyme, however, were also observed in the light and heavy mitochondrial fractions, 34 and 8.8 per cent of the microsomal specific activity, respectively. This contamination of the mitochondrial fractions by the microsomal fraction can be reduced with an increasing number of washes, particularly in the heavy mitochondrial fraction. After three washes only 4–5 per cent of both the microsomal cytochrome P-450 and NADPH–cytochrome *c* reductase were observed in the heavy mitochondrial fraction in comparison to 9–10 per cent with two washes. With repeated washings, however, a decrease in total yield of the mitochondrial fraction was observed. This microsomal contamination of mitochondria was not crucial since *trans*-β-alkylstyrene oxides, poor substrates for the microsomal epoxide hydrolase [18, 28], were used to monitor epoxide hydrolase activity in most experiments. Thus, in routine experiments, mitochondria were washed only twice.

Cytochrome oxidase activity was recovered predominatly in the heavy mitochondrial and cell nuclei and debris fractions (Table 1). This activity in the cell nuclei and debris fraction was probably due to contamination by mitochondria or incomplete disruption of cellular material. A low level of cytochrome oxidase was found in the light mitochondrial fraction. Low cytochrome oxidase levels in the microsomal and cytosolic fractions indicated minimal contamination by intact mitochondria.

Lactate dehydrogenase, a marker enzyme for the cytosol, was predominantly located in the 100,000 *g* supernatant fraction (Table 1). Low levels of activity were recovered in the cell nuclei and debris fraction with lesser activity in the other fractions. In contrast, acid phosphatase activity, monitored by *p*-nitrophenyl phosphate, was quite evenly distributed in the subcellular fractions, with the highest levels recorded in the light and heavy mitochondrial fractions. Significant levels of activity were also found in the microsomal and cell nuclei and debris fractions. The widespread distribution of *p*-nitrophenyl phosphate hydrolyzing activity was probably encountered due to the presence of a variety of enzymes that are able to utilize *p*-nitrophenyl phosphate as substrate. For instance, phosphatases present in the endoplasmic reticulum are known to hydrolyze *p*-nitrophenyl phosphate [29]. This marker proved very useful, however, in distinguishing between mitochondrial-rich and lysosomal-rich fractions.

Epoxide hydrolase activity in subcellular fractions in normal mice. Subcellular distribution of epoxide hydrolase activity in male Swiss–Webster mouse liver varied with the substrate used for monitoring activity. With benzo[*a*]pyrene 4,5-oxide, the highest level of activity was found in the microsomal fraction (Table 1). Significant levels of activity were also present in the light and heavy mitochondrial fractions, and in the cell nuclei and debris fraction. Activity in the mitochondrial fractions was probably due to contamination by the microsomal or nuclei

Table 1. Distribution of epoxide hydrolase activity and marker enzymes in subcellular fractions of normal Swiss–Webster male mice*

Subcellular fraction	Total protein (mg)	NADPH–cytochrome *c* reductase	Cytochrome oxidase	Acid phosphatase	Lactate dehydrogenase	Epoxide hydrolase†		
						trans-β-Ethylstyrene oxide	*trans*-Stilbene oxide	Benzo[*a*]pyrene 4,5-oxide
Cell nuclei and debris	270	7.0	82	23	160	16	1.9	1.7
Heavy mitochondria	160	10	96	24	90	49	3.4	1.3
Light mitochondria	41	41	7.2	35	15	57	4.6	2.3
Microsomes	87	118	3.1	21	30	5.0	0.5	7.7
Soluble	320	1.2	0.2	11	4100	79	6.3	0.2

* All enzyme activities are in nmoles · min^{-1} · (mg protein) $^{-1}$. Values reported are averages of a minimum of two to three determinations from a preparation using three mouse livers; the experiment was repeated three times. Though there was variation between preparations, the relative distribution of enzyme activities was similar. This differential centrifugation scheme was designed to yield highly enriched mitochondrial fractions at the possible expense of mitochondrial yield and contamination of the cell nuclei and debris fraction with some mitochondria.

† Epoxide hydrolase activity was monitored at 37° in sodium phosphate buffer, pH 7.4, ionic concentration (I) = 0.2 M, for *trans*-β-ethylstyrene oxide and *trans*-stilbene oxide and in Tris–HCl buffer, pH 9.0, I = 0.1 M, for benzo[*a*]pyrene 4,5-oxide.

and cell debris fractions as indicated by the presence of NADPH–cytochrome *c* reductase (Table 1). With *trans*-β-ethylstyrene oxide, however, the highest level of activity was recovered in the 100,000 *g* soluble fraction or the cytosol. Significant levels of activity were also found in the light and heavy mitochondrial fractions. The specific adenylate kinase and citrate synthase activities of the cytosolic fraction were greater than fifty and two hundred times lower, respectively, than those of the mitochondrial matrix and intermembrane space, indicating that the majority of the cytosolic epoxide hydrolase activity did not arise from mitochondrial lysis during homogenization. Of the total *trans*-β-ethylstyrene oxide hydrolase activity recovered in the various subcellular fractions, 11, 20, 5.8, 1.1 and 63 per cent of the activity was present in the cell nuclei and debris, heavy and light mitochondrial, microsomal, and cytosolic fractions, whereas with benzo[*a*]pyrene 4,5-oxide the same fractions had 31, 14, 6.3, 45 and 4.3 per cent. With *trans*-β-ethylstyrene oxide and benzo[*a*]pyrene 4,5-oxide, 70 and 78 per cent, respectively, of the total epoxide hydrolase activity in the crude cell homogenate were recovered in the subcellular fractions. The discarded washings of the subcellular fractions probably accounted for a significant amount of the epoxide hydrolase activity lost with both substrates.

With *trans*-stilbene oxide as substrate, the subcellular distribution of epoxide hydrolase activity was very similar to that observed with *trans*-β-ethylstyrene oxide (Table 1). In contrast, its geometrical isomer, *cis*-stilbene oxide, shows a pattern of distribution more akin to that observed with benzo[*a*]pyrene 4,5-oxide.

Subcellular distribution of marker enzymes and epoxide hydrolase activity in mice treated with Triton. Pretreatment of rats with Triton WR-1339 is reported to result in the selective uptake of the Triton by the liver lysosomes, giving rise to a selective decrease in lysosomal equilibrium density in a sucrose gradient [20]. Thus, an attempt was made to utilize this technique for lysosomal separation in subcellular fractions. Mice treated with Triton WR-1339 had livers with weights similar to that of normal livers. These livers, however, were a lighter brown than normal livers. Homogenates prepared from such livers gave a subcellular distribution of marker enzymes and epoxide hydrolase activity quite similar to that of normal livers (Table 2). However, higher levels of cytochrome oxidase were encountered in the cell nuclei and debris and mitochondrial fractions, probably due to the presence of administered Triton WR-1339 in these fractions. Schnaitman and Greenawalt [30] reported similar values for cytochrome oxidase in rat preparations activated with Lubrol WX.

Epoxide hydrolase specific activity was highest in the light mitochondrial fraction, with high levels in the heavy mitochondrial and cytosolic fractions. The greatest amount of total epoxide hydrolase activity present, however, was in the cytosolic fraction. Of the total epoxide hydrolase activity recovered, 8.4, 17, 7.8, 4.0 and 63 per cent were in the cell nuclei and debris, heavy and light mitochondrial, microsomal and cytosolic fractions, respectively, showing a pattern of distribution similar to that of untreated mice.

The heavy and light mitochondrial fractions were layered separately onto discontinuous sucrose gradients and were centrifuged to separate mitochondria and lysosomes. Two distinct layers, an upper lysosomal-rich and a lower mitochondrial-rich layer, were obtained in both cases, as shown for the heavy mitochondria (Table 3). Distribution of marker enzymes shows that mitochondria and lysosomes can be distinctly separated. A 4.9 per cent mitochondrial contamination of the lysosomal fraction and a 3.6 per cent lysosomal contamination of the mitochondrial fraction were observed, based on marker enzyme specific activities. Table 3, however, illustrates that peroxisomes, as indicated by urate oxidase activity, and mitochondria do not separate. Similar results were obtained by Leighton *et al.* [20], although with a more complex procedure they were subsequently able to separate lysosomes, mitochondria and peroxisomes.

Epoxide hydrolase activity as monitored by *trans*-β-ethylstyrene oxide was present predominantly in the mitochondrial fraction. Only 0.1 per

Table 2. Distribution of epoxide hydrolase and marker enzymes in subcellular fractions of Triton WR-1399-treated Swiss–Webster male mouse liver*

Subcellular fraction	Total protein (mg)	NADPH–cytochrome *c* reductase	Cytochrome oxidase	Acid phosphatase	Lactate dehydrogenase	Urate oxidase	*trans*-β-Ethylstyrene oxide hydrolase
Cell nuclei and debris	360	17	1600	13	280	57	13
Heavy mitochondria	220	13	2000	7.7	64	240	43
Light mitochondria	46	23	180	27	96	630	94
Microsomes†	170	72	40	16	270	60	13
Soluble	580	2.7	ND‡	5.0	2500	9.3	60

* Enzyme activities are expressed in nmoles · min^{-1} · (mg protein)$^{-1}$ and are average values of two to four determinations from a preparation using four mice.

† Microsomes were not washed.

‡ Not detectable.

Table 3. Distribution of epoxide hydrolase and marker enzymes in the 6,500 *g* pellet of Triton WR-1339-treated Swiss–Webster male mouse liver after differential centrifugation in a discontinuous sucrose gradient*

Subcellular fraction	Total protein (mg)	NADPH–cytochrome *c* reductase	Cytochrome oxidase	Acid phosphatase	Lactate dehydrogenase	Urate oxidase	Epoxide hydrolase	
							trans-β-Ethylstyrene oxide	*trans*-Stilbene oxide
Mitochondrial	140	27	2500	8.6	100	1000	59	5.4
Lysosomal	5.6	5.4	130	230	47	110	1.0	0.1

* Enzyme activities are expressed in nmoles·min^{-1}·(mg protein)$^{-1}$ and are average values of a minimum of two to three determinations from a preparation using four mice. For this preparation, 190 mg of protein from the heavy mitochondrial fraction described in Table 2 was used. Essentially all epoxide hydrolase activity (101 per cent) was recovered in the two fractions shown above. The additional protein was spread among other fractions of the sucrose gradient. Very similar results were obtained with the light mitochondrial fraction.

cent of the total recovered epoxide hydrolase activity was present in the lysosomal fraction. Though it was not possible to separate peroxisomal and mitochondrial fractions totally, it was observed during sucrose density gradient separations that peroxisome activity was present predominatly (97 per cent) in the bottom portion of the mitochondrial fraction. In contrast, a greater amount of the epoxide hydrolase activity (72 per cent) was in the upper portion of the mitochondrial fraction. This partial separation of peroxisomes and mitochondria showed that epoxide hydrolase activity was largely associated with the mitochondria.

Distribution of marker enzymes and epoxide hydrolase activity in the submitochondrial components. Separation of submitochondrial components in a discontinuous sucrose density gradient resulted in a visual separation similar to that reported by Sottocassa *et al.* [19]. Of the five distinct fractions collected, the three major ones are an upper layer corresponding to the matrix and intermembrane space, an intermediate layer directly beneath the upper layer consisting of the outer membrane, and an inner membrane pellet. Distribution of marker enzymes and epoxide hydrolase activity in these three submitochondrial fractions (Table 4) shows that a separation such as that reported by Sottocassa *et al.* [19] for the rat was obtained, although no marker enzyme was run for the outer membrane. Absolute activities in the rat, however, differ from those reported here for the mouse. NADPH–cytochrome *c* reductase, lactate dehydrogenase and acid phosphatase values were followed to show possible percent contamination of the mitochondrial fractions by the microsomal, cytosolic and lysosomal components, respectively. The highest level of epoxide hydrolase activity was recovered in the matrix and intermembrane space fraction. Significant epoxide hydrolase activity was also observed in the outer and inner membrane fractions. Most of the specific activity in these two fractions (83 and 88 per cent, respectively), however, could be removed with a single resuspension and recentrifugation, indicating probable contamination of these fractions by the matrix and intermembrane space fraction. The results of the subcellular and submitochondrial distribution studies (Tables 1–4) thus show that a significant amount of epoxide hydrolase activity was present in the matrix and/or intermembrane space.

Mitochondrial epoxide hydrolase activity in rat liver. Subcellular and submitochondrial separations demonstrated only low levels of mitochondrial epoxide hydrolase activity in rat liver, using *trans*-β-ethylstyrene oxide (Table 5). Using *trans*-stilbene oxide as substrate, epoxide hydrolase activity in the 6,500 *g* and 12,000 *g* pellets was not detected, and very low levels were detected in submitochondrial fractions. In contrast, with benzo[*a*]pyrene 4,5-oxide high levels of epoxide hydrolase activity were found in the nuclear and cell debris, light mitochondrial and microsomal fractions. Activity in the light mitochondrial fraction was largely due to microsomal contamination since the fraction was washed only once and significant microsomal contamination occurred.

Hydration of various epoxides by the mouse mito-

Table 4. Distribution of epoxide hydrolase and marker enzymes in fractions of the 6,500 *g* pellet of normal Swiss–Webster mouse liver after swelling-contraction-sonication and differential centrifugation in a discontinuous sucrose gradient*

Submitochondrial fraction	Total protein (mg)	NADPH–cytochrome *c* reductase	Cytochrome oxidase	Acid phosphatase	Lactate dehydrogenase	Adenylate kinase†	Citrate synthase†	Epoxide hydrolase		
								trans-β-Ethylstyrene oxide†	*trans*-Stilbene oxide	Benzo[*a*]-pyrene 4,5-oxide
Matrix and intermembrane space	23	4.6	9.6	16	100	1000	443	104	7.5	0.6
Outer membrane	8.4	20	69	38	190	660	256	43	2.4	2.3
Inner membrane	57	11	90	20	150	220	360	42	2.8	1.3

* Values are expressed in nmoles · min^{-1} · (mg protein)$^{-1}$ and are averages of a minimum of two to four determinations of a preparation using three mice. Values between preparations varied but relative values are similar. For this preparation 95 mg of protein from the heavy mitochondrial fraction described in Table 1 was used. All the *trans*-β-ethylstyrene oxide hydrolase activity (110 per cent) and 93 per cent of the total protein used were recovered in the three fractions shown above. The rest of the protein (7 per cent) containing 1 per cent of the epoxide hydrolase activity was recovered in the 1.18 M sucrose fraction.

† Increased sonication times lead to an increase in enzyme activity in the matrix fraction at the expense of the membrane-bound fractions. Activity in the latter fractions could also be reduced by washing and recentrifugation.

chondrial epoxide hydrolase. The mouse mitochondrial epoxide hydrolase was able to hydrate a variety of substrates (Table 4). *trans*-β-Ethylstyrene oxide, methyl *cis*-epoxystearate and *trans*-stilbene oxide were rapidly hydrated, whereas hydration of the ethyl epoxide was relatively slower. *cis*-Stilbene oxide, allylbenzene oxide, styrene oxide and benzo[*a*]pyrene 4,5-oxide were, in contrast, poor substrates. The highest specific activities of *cis*-stilbene oxide and benzo[*a*]pyrene 4,5-oxide hydrolase were greatest in the outer membrane fraction. This observation supports the theory of Oesch [2] that the minimal level of epoxide hydrolase activity in the mitochondrial fraction when styrene oxide is used as a substrate is due largely to microsomes adhering to the mitochondrial membranes. The very low relative rates of hydration of these epoxides were similar in the matrix and inner membrane fractions. In general, epoxides that are good substrates of the microsomal and nuclear epoxide hydrolase, such as benzo[*a*]pyrene 4,5-oxide and styrene oxide [2], were poorly hydrated by the mitochondrial epoxide hydrolase, but epoxides that are good substrates of the cytosolic epoxide hydrolase were hydrated rapidly by the mitochondrial epoxide hydrolase.

Gel filtration and inhibition of the mitochondrial epoxide hydrolase. Gel filtration profiles were consistent with the mitochondrial epoxide hydrolase having a molecular weight between 120,000 and 140,000 daltons, and the elution volumes of the epoxide hydrolase activities in the mitochondrial and cytosolic fractions were indistinguishable on Sephacryl S-200. Similarly, a variety of compounds, such as chalcone oxide, hydroxychalcone, 2-bromo-4′-nitroacetophenone and 5,5′-dithiobis(2-nitrobenzoic acid), which act as good inhibitors of the cytosolic epoxide hydrolase, also serve as good inhibitors of the mitochondrial epoxide hydrolase.

DISCUSSION

The results of the subcellular distribution studies (Tables 1–3) show that four enzyme markers for various organelles of mouse liver cells were usually distributed as published reports indicate, following differential centrifugation [19, 30–33]. The cytosolic fraction of mouse liver had little contamination from the various subcellular particulate-bound enzymes (Table 1) or with enzymes from the matrix or intermembrane space of lysed mitochondria. This information, along with earlier published reports [13, 14], further indicates that the cytosolic epoxide hydrolase encountered in mouse liver does not result from dissociation or solubilization of the endoplasmic reticulum or other particulate fractions.

The subcellular distribution studies further demonstrate that a significant amount (25 per cent) of total *trans*-β-ethylstyrene oxide hydrolase activity recovered in subcellular fractions was present in the light and heavy mitochondrial fractions. This activity in the mitochondria consisted of the second largest component of epoxide hydrolase activity in the cell. Epoxide hydrolase activity in the mitochondria is not a result of contamination from either of the three major, presently known sources of epoxide hydro-

Table 5. Epoxide hydrolase activity of subcellular and submitochondrial fractions of Sprague–Dawley male rat liver*

Fraction	*trans*-β-Ethylstyrene oxide	*trans*-Stilbene oxide	Benzo[*a*]pyrene 4,5-oxide
Cell nuclei and debris	0.7 ± 0.1	ND†	4.0 ± 0.3
Heavy mitochondria	0.3 ± 0.1	ND	0.6 ± 0.02
Light mitochondria	2.0 ± 1.0	ND	9.5 ± 0.2
Microsomes	2.0 ± 1.0	ND	15.0 ± 3.0
Cytosol	5.7 ± 2.4	2.6 ± 0.2	0.1 ± 0.01
Heavy mitochondrial sub-fractions			
Matrix and intermembrane space	1.8 ± 0.1	ND	
Outer membrane	1.0 ± 0.1	0.1 ± 0.02	
Inner membrane	0.5 ± 0.1	ND	

* Values are expressed in nmoles · min^{-1} · $(mg\ protein)^{-1}$ and are averages of a minimum of two to four determinations of a preparation using two rats.
† Not detectable.

lase: the nuclear membrane, microsomal and cytosolic fractions. Table 1 illustrates that microsomal and cytosolic contamination was minor, as indicated by NADPH–cytochrome *c* reductase and lactate dehydrogenase levels, respectively. Similarly, the nuclear fraction is unlikely to have been a major contaminant because the speed of centrifugation used (600 *g* for 10 min) generally sediments most of the nuclei. In fact, at this speed, significant amounts of the mitochondria were precipitated as indicated by cytochrome oxidase (Table 1) [20]. Further, by using benzo[*a*]pyrene 4,5-oxide hydrolase as an enzyme marker for nuclear membranes [15], one can conclude that its relative presence in the cell nuclei and debris and mitochondrial fractions (Table 1) does not explain the epoxide hydrolase activity encountered in the mitochondrial fraction. Since *trans*-β-alkylstyrene oxides are poor substrates for the microsomal epoxide hydrolase [18, 28], they are also not likely to be good substrates for the immunologically similar nuclear epoxide hydrolase [16]. Thus, epoxide hydrolase activity present in mitochondria was probably not due to contamination by nuclei.

Differential centrifugation did not separate lysosomes and peroxisomes from mitochondria in normal Swiss–Webster mouse liver (Table 1). However, *trans*-β-ethylstyrene oxide hydrolase activity was shown to be absent from the lysosomal fraction in enzyme preparations from Triton WR-1339-treated mice (Tables 2 and 3). Peroxisomes are also not likely to be the site of high epoxide hydrolase activity, although more careful studies should be done to separate peroxisomes and mitochondria. It is, therefore, reasonable to conclude that the epoxide hydrolase activity encountered in the 6,500 *g* and 12,000 *g* fractions was present largely in the mitochondria.

Within the mitochondria, epoxide hydrolase activity occurred predominantly in the matrix and/or intermembrane space (Table 4), which could not be separated by the procedures reported here. The epoxide hydrolase activity encountered in the inner and outer membrane fractions of mitochondria may have resulted from contamination by proteins from the matrix/intermembrane space. The absence of any previous report demonstrating epoxide hydrolase activity in the mitochondria of mammals is due, in part, to other investigators using either styrene oxide or benzo[*a*]pyrene 4,5-oxide as substrate and the rat liver as an enzyme source [9, 15, 16, 34, 35]. The data presented here and elsewhere [14, 28, 36] show that, using these substrates and rats, it is unlikely that mitochondrial epoxide hydrolase would have been encountered. With styrene and benzo[*a*]pyrene 4,5-oxide as substrates, epoxide hydrolase activity occurs predominantly in the endoplasmic reticulum and the nuclear membrane [9, 15, 16, 35], whereas with ethyl epoxide, methyl *cis*-epoxystearate, *trans*-β-ethylstyrene oxide and *trans*-stilbene oxide, maximal activity is observed in the cytosolic fraction, with significant levels occurring in the mitochondria [13, 14, 37]. Nuclear and microsomal epoxide hydrolases occur in higher levels in rats than in mice. The reverse relationship, however, holds true for cytosolic and mitochondrial epoxide hydrolases [6, 13, 14, 28]. Thus, attempts to delineate subcellular distribution of epoxide hydrolase activity must be qualified with the substrate and animal species utilized.

The mitochondrial epoxide hydrolase appears similar, in some respects, to the cytosolic epoxide hyrolase. Both enzymes have similar substrate selectivities (Table 4) [13, 14, 26, 28] and molecular weights [14], and they are inhibited by the same

compounds. This similarity of the cytosolic and mitochondrial epoxide hydrolases might be expected since it is generally recognized that mitochondrial and cytoplasmic protein-synthesizing systems are tightly coupled [38].

A major biological function of any of the epoxide hydrolases in mammalian liver and other tissues has not yet been unequivocally demonstrated, and the mitochondrial epoxide hydrolase is no exception. In addition to possible involvement in some aspects of intermediary metabolism, these enzymes could be involved in the metabolism of potentially damaging epoxidized compounds. Such epoxides in mitochondria can possibly result from free radicals formed during normal respiration or from oxidation of olefinic and aromatic compounds by mitochondrial oxygenases. Epoxide metabolizing activity in mitochondria may be crucial since epoxidized natural products or xenobiotics could disrupt membrane integrity or alkylate biologically significant molecules. For instance, Allen and Coombs [39] recently reported that mouse mitochondrial DNA is exceptionally susceptible to alkylation by several polycyclic aromatic hydrocarbons, presumably after activation by oxidases on the inner or outer mitochondrial membrane [40, 41]. Since epoxide hydrolases capable of hydrating arene oxides are reported to be largely absent from mitochondrial membranes and matrix epoxide hydrolase, just as the cytosolic epoxide hydrolase [36] does not appear to rapidly hydrate arene oxides, such results might be anticipated. However, the epoxide hydrolases in the mitochondrial matrix could protect mitochondrial DNA from other reactive epoxides that could serve as substrates.

As judged from previous literature, epoxide metabolizing systems appear to be ubiquitous in mammalian liver cells. GSH transferases occur in high levels in the cytosol [5] and, in addition, GSH transferases occur in the mitochondrial and microsomal membranes [42–44]. Epoxide hydrolase activity has been reported previously in the cytosol and in the microsomal and nuclear membranes [2, 11, 14, 15]. Thus, this report of epoxide hydrolase activity in the mitochondrial fraction is not altogether surprising.

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