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

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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 100,000g 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-14C-phenoxy]-3,7-dimethyl-6,7-epoxy-2E-octene (ethyl epoxide, 0.63 GBq/mmol, > 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-epoxystearate was synthesized from [1-14C]-oleic acid (1.1 GBq/mmol,
containing 2 mM adenosine-triphosphate and 2 mM 
MgSO₄ was added. After an additional 5 min, the 
mitochondrial fraction was resuspended and repel-
trifuged at 600g for 10 min. The precipitate was 
removed and the pellet was resuspended in 
0.1% Triton X-100 according to the method of 
Sottocassa et al. [19]. Cytochrome P-450, similarly an endo-
plasmic 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 endo-
plasmic reticulum marker, was estimated by the 
CO-difference spectra of sodium dithionite-reduced 
matrix and intermembrane space, the outer mito-
chondrial membrane layer, an intermediate 1.18 M 
sucrose layer, and the inner mitochondrial mem-
brane 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 
for 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 homog-
ized as indicated earlier and the various subcel-
lular 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 lay-
ted on top. The tube was then centrifuged at 
80,000 g for 2 hr in a SW 41 rotor. Two distinct 
fractons 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 
fractons were diluted with buffer to give appropriate 
protein concentrations before use. Protein concen-
tration 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 submi-
 tochondrial fractions. The inner mitochondrial mem-
brane 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 endo-
plasmic 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-nitro-
phenyl phosphate as substrate according to Berg-
meyer [23] in the presence of 0.3% Triton X-100. 
Enzyme assays were performed after fractions were 
incubated for 5 min in 50 mM phosphate 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 acid esters, with appropriate internal standards. Unless otherwise indicated, the following concentrations of epoxides and incubation times were used: trans-β-ethyldistyrene 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 × 10⁻⁴ M, 10 min), and p-chlorophenoxyepoxypropane (5 × 10⁻⁴ 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 non-denatured protein concentrations (1–20 µg/assay) with incubation time.

Molecular weight estimation. Mitochondrial epoxide hydrolase molecular weight was estimated using a Sephacyr 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 IC₅₀ 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°C 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 predominantly 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 subcellar 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.

Epoxide hydrolase activity in mouse liver
Table 1. Distribution of epoxide hydrolase activity and marker enzymes in subcellular fractions of normal Swiss-Webster male mice

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Total protein (mg)</th>
<th>NADPH-cytochrome c reductase</th>
<th>Cytochrome oxidase</th>
<th>Acid phosphatase</th>
<th>Lactate dehydrogenase</th>
<th>trans-1-Ethylstylene oxide</th>
<th>trans-Stilbene oxide</th>
<th>Benzo[a]pyrene 4,5-oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell nuclei and debris</td>
<td>270</td>
<td>7.0</td>
<td>82</td>
<td>23</td>
<td>160</td>
<td>16</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Heavy mitochondria</td>
<td>160</td>
<td>10</td>
<td>96</td>
<td>24</td>
<td>90</td>
<td>49</td>
<td>3.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Light mitochondria</td>
<td>41</td>
<td>41</td>
<td>7.2</td>
<td>35</td>
<td>15</td>
<td>57</td>
<td>4.6</td>
<td>2.3</td>
</tr>
<tr>
<td>Microsomes</td>
<td>87</td>
<td>118</td>
<td>3.1</td>
<td>21</td>
<td>30</td>
<td>5.0</td>
<td>0.5</td>
<td>7.7</td>
</tr>
<tr>
<td>Soluble</td>
<td>320</td>
<td>1.2</td>
<td>0.2</td>
<td>11</td>
<td>4100</td>
<td>70</td>
<td>6.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* 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°C in sodium phosphate buffer, pH 7.4, ionic concentration (I) = 0.2 M, for trans-1-Ethylstylene 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-ethylstylene 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-ethylstylene 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-ethylstylene 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

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Total protein (mg)</th>
<th>NADPH-cytochrome c reductase</th>
<th>Cytochrome oxidase</th>
<th>Acid phosphatase</th>
<th>Lactate dehydrogenase</th>
<th>Urate oxidase</th>
<th>trans-β-Ethylstylene oxide hydrolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell nuclei and debris</td>
<td>260</td>
<td>17</td>
<td>1600</td>
<td>13</td>
<td>280</td>
<td>57</td>
<td>13</td>
</tr>
<tr>
<td>Heavy mitochondria</td>
<td>220</td>
<td>13</td>
<td>2000</td>
<td>7.7</td>
<td>64</td>
<td>240</td>
<td>43</td>
</tr>
<tr>
<td>Light mitochondria</td>
<td>46</td>
<td>23</td>
<td>180</td>
<td>27</td>
<td>96</td>
<td>630</td>
<td>94</td>
</tr>
<tr>
<td>Microsomes†</td>
<td>170</td>
<td>72</td>
<td>40</td>
<td>16</td>
<td>270</td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td>Soluble</td>
<td>580</td>
<td>2.7</td>
<td>ND‡</td>
<td>5.0</td>
<td>2500</td>
<td>9.3</td>
<td>60</td>
</tr>
</tbody>
</table>

* 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.
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... 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 predominantly (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) 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-p-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.

**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.**

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Mitochondrial</th>
<th>Lysosomal</th>
<th>NADPH-cytochrome c reductase</th>
<th>Cytochrome c oxidase</th>
<th>Acid phosphatase</th>
<th>Lactate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (mg)</td>
<td>14.9</td>
<td>5.6</td>
<td>2.7</td>
<td>8.6</td>
<td>100</td>
<td>130</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>13.0</td>
<td>5.4</td>
<td>2.4</td>
<td>7.6</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Lysosomal</td>
<td>1.9</td>
<td>1.2</td>
<td>0.3</td>
<td>0.4</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

* Enzyme activities are expressed in units/min/mg protein (mg protein)−1 and are average values of two to three determinations from a preparation of liver homogenate. The activity (units/min/mg protein) was recovered in the two fractions showing the highest activity. The additional protein was present among other fractions of the sucrose gradient. Very similar results were obtained with the light mitochondrial fraction.
The mouse mitochondrial epoxide hydrolase was able to hydrate a variety of substrates (Table 4). trans-β-Ethylstyrene oxide, methyl cis-epoxysestearate 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 hydrolase activity.
Table 5. Epoxide hydrolase activity of subcellular and submitochondrial fractions of Sprague-Dawley male rat liver

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

* 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.

...
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 alkylolation by several polyyclic aromatic hydrocarbons, presumably after activation by oxides 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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