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PREPARATION AND CHARACTERIZATION OF SUBCELLULAR FRACTIONS FROM THE LIVER OF C57B1/6 MICE, WITH SPECIAL EMPHASIS ON THEIR SUITABILITY FOR USE IN STUDIES OF EPOXIDE HYDROLASE ACTIVITIES

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Abstract—The present study was designed to prepare and characterize subcellular fractions from the liver of male C57B1/6 mice, with special emphasis on their suitability for use in studies of epoxide hydrolase isozymes. The effects of different washing and pelleting procedures on the mitochondrial, microsomal and cytosolic fractions were studied. It was found that 133,000 g_{av} for 60 min (i.e. more extensive force than the usual 105,000 g_{av} for 60 min) was necessary to obtain a membrane-free cytosolic fraction, while one wash for microsomes and two washes for mitochondria yielded reasonably pure fractions.

The purity of the different fractions obtained by differential centrifugation was then determined using established enzyme markers and morphological examination with the electron microscope. Several enzymes involved in drug metabolism were also measured in these fractions. The subcellular distributions obtained here for marker enzymes closely resemble those reported for rat liver.

Starvation had no significant effect on the epoxide hydrolase activities nor did the addition of mouse bile or rat liver cytosol, which might contain inhibitors. The change in epoxide hydrolase activities with time after preparation of the subcellular fractions was studied, as well as the effect of freeze-thawing. The subfractions prepared here are suitable for the further characterization of the different forms of epoxide hydrolase present in mouse liver, as well as for other studies requiring well-characterized subfractions.

Living organisms in industrial societies are exposed to an enormous number of different xenobiotics. The Environmental Protection Agency has calculated the number of chemicals in "daily use" in the United States to be about 63,000. A major route of exposure is in the diet and xenobiotics may be taken up in the intestine. The blood flow from the intestine is directed towards the liver, where drug-metabolizing enzymes are present in relatively high levels [1]. If chemicals pass the liver without being taken up and/or metabolized (and some metabolites are even released from the liver), they can also be metabolized by other organs, which, however, usually have much lower enzyme activities [2].

In the hepatocyte xenobiotic-metabolizing enzymes are localized to different cellular compartments, e.g. the cytochrome P-450 system mainly to the endoplasmic reticulum [3] and sulfotransferases mainly to the cytosol [4]. Some of these enzymes are found, often as different isozymes, in several compartments, e.g. glutathione transferases and epoxide hydrolases in the cytosol, microsomes and mitochondria [5, 6]. Although the functional significance of these differences in subcellular localization are not really understood, such patterns have probably evolved to optimize detoxication and/or reflect endogenous functions of these same enzymes.

Epoxide hydrolases metabolize epoxides to the corresponding dihydrodiols through the addition of water and thereby transform more-or-less reactive electrophiles to more harmless products which may be excreted either directly or, more often, after further metabolism, in the urine or the bile [4, 7]. Some dihydrodiols may, however, be recycled through the cytochrome P-450 system, giving rise to more reactive intermediates [8].

On the basis of studies with rat liver, epoxide hydrolase activity was first thought to be localized solely on the endoplasmic reticulum in hepatocytes [9]. Later on, cytosolic and mitochondrial epoxide hydrolase activities were found in several other species, such as mouse, guinea pig, hamster and rabbit, which all exhibit easily measurable levels in these two other fractions as well [10–12; unpublished]. The rat, however, has very low levels of cytosolic and mitochondrial epoxide hydrolase activity [10, 11], a difference the functional significance of which remains to be elucidated.

Since epoxide hydrolase activity can be found in several subcellular fractions, it is important to ascertain whether this reflects localization in different organelles or merely cross-contamination. One approach to this question is analytical subfractionation. Rat liver has been subfractionated analytically by several researchers and different methods to prepare pure organelles have also been developed [13].

Since we are interested in studying all known isozymes of epoxide hydrolase, we have chosen to

work with mouse liver. Mice are also easy to handle in induction experiments and there are a multitude of different strains available. To our knowledge, no detailed analytical subfractionation has yet been performed with mouse liver, so we felt it important to do an initial study to characterize different subcellular fractions from this tissue.

Here we present data on the purity, based on enzyme markers and morphological criteria, of different subcellular fractions obtained by differential centrifugation of mouse liver homogenate. The subcellular distributions of certain xenobiotic-metabolizing enzymes, especially epoxide hydrolases, have also been examined. Furthermore, the effects of starvation, addition of bile, freeze-thawing and time of storage of the subcellular fractions on epoxide hydrolase activities were also investigated. Some of the results presented here have appeared earlier in preliminary form [14].

MATERIALS AND METHODS

Chemicals. 1-Chloro-2,4-dinitrobenzene and hydrogen peroxide (Merck, Darmstadt, F.R.G.), AMP, cytochrome *c*, deoxyribonucleic acid (salmon sperm), glutathione, glucose-6-phosphate, β-glycerophosphate, pyruvic acid, *p*-nitrophenyl-α-mannoside, NADH and NADPH (Sigma Chemical Co., St. Louis, MO) and *trans*-stilbene oxide (EGA-Chemie, Steinheim, F.R.G.) were all purchased from the sources indicated.

cis-Stilbene oxide was synthesized by *m*-chloroperoxybenzoic acid oxidation of *cis*-stilbene (Merck, Darmstadt, F.R.G.) and purified before use by silica gel column chromatography with hexane/ethylacetate (95:5, v/v) as the solvent system. *cis*-[^{3}H]Stilbene oxide and *trans*-[^{3}H]stilbene oxide were synthesized at a specific radioactivity of approximately 2 Ci/mmol (74GBq/mmol) as described [15] and purified before use to >99% by thin-layer chromatography on 0.2 mm silica gel plates (Merck, Darmstadt, F.R.G.) with hexane/ethylacetate (95:5, v/v) as the eluant. [7-^{3}H]Styrene oxide (10 Ci/mol = 370 GBq/mol) (the Radiochemical Centre, Amersham, U.K.) was purified using an extraction procedure [16].

Animals. Male C57B1/6 mice (20–25 g) and Sprague–Dawley rats (180–200 g) were obtained from ALAB (Sollentuna, Sweden). The animals were housed in groups of 3–5 in wire-bottomed cages with a 12-hour-light/12-hour-dark cycle and free access to water and a commercial rat diet (R-3 from Ewos AB, Södertälje, Sweden, containing 5% fat, 24% protein and 49% carbohydrates).

Subcellular fractionation. The animals were generally starved overnight before sacrifice by cervical dislocation and the livers excised and, in the case of the mice, gallbladders removed. All subsequent procedures were then performed at 0–4°. The livers were weighed, placed in ice-cold 0.25 M sucrose, rinsed, cut into small pieces with scissors and then homogenized using 4 up-and-down strokes in a Potter-Elvehjem homogenizer at 440 rpm.

This homogenate was subsequently diluted to 20% (1 g tissue/5 ml) with sucrose and centrifuged for 10 min at 600 g_{av}. The resulting pellet was resuspended to the same volume as the original homogenate and centrifuged again and this procedure was repeated once again. The final 600 g_{av} pellet was resuspended in 0.25 M sucrose and designated the nuclear fraction.

The supernatant from the first 600 g_{av} centrifugation was centrifuged for 10 min at 10,000 g_{av} and the resulting pellet washed twice in a manner analogous to that described above. The final 10,000 g_{av} pellet was resuspended in 0.25 M sucrose and designated the mitochondrial fraction.

The supernatant from the first 10,000 g_{av} centrifugation was centrifuged for 60 min at 105,000 g_{av}. The resulting pellet was resuspended in 0.15 M Tris–Cl, pH 8.0, and centrifuged for 30 min at 105,000 g_{av} in order to remove adsorbed cytoplasmic protein [17]. The washed pellet was resuspended in 0.25 M sucrose and designated the microsomal fraction. The supernatant from the first 105,000 g_{av} centrifugation was designated the cytosolic fraction.

The cytosolic fraction was also centrifuged at 133,000 g_{av} for 60 min and the resulting supernatant fraction, as well as the original cytosolic fraction, were centrifuged at 246,000 g_{av} for 120 min in some experiments.

Aliquots were taken immediately from each fraction, flushed with nitrogen, capped and stored at −20° or −70° for investigation of the effects of freeze-thawing and storage.

Chemical and enzyme assays. All assays were originally performed on freshly prepared subfractions. Membrane fractions were not sonicated before use. Cytochrome oxidase and glutathione transferase activities in the mitochondrial fraction were found to decrease after such treatment. In the assays using a hydrophilic substrate a detergent was used routinely, whereas the hydrophobic substrates are expected to penetrate membranes easily.

AMPase [18], catalase [19], cytochrome oxidase [20], DNA [21], epoxide hydrolase activity towards *cis*- and *trans*-stilbene oxide [15] and with styrene oxide [16], glucose 6-phosphatase (with 0.03% DOC) [22], glutathione transferase activity (with 5 mM glutathione in the case of membraneous fractions and 1 mM glutathione for the cytosolic fraction with 1-chloro-2,4-dinitrobenzene, as the second substrate [23] and with 5 mM glutathione when *cis*- or *trans*-stilbene oxide [15] was the second substrate), β-glycerophosphatase [24], lactate dehydrogenase [25], NADPH-cytochrome *c* reductase [26], *p*-nitrophenyl-α-mannosidase [27] and protein [28] with bovine serum albumin as standard were all determined according to previously published procedures.

deDuve plots. The distributions of different enzymes in the subcellular fractions were expressed as deDuve plots [29]. The results from either 3 different experiments with livers pooled from 5 mice or from 4 mice treated individually are shown and the standard deviations indicated by bars in the plots.

Electron microscopy. An aliquot of the subcellular fraction (pooled from 4 individually treated animals) was added to 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) with 3.42% sucrose and fixed at 0°.

After centrifugation and washing, the pellets were fixed with 2% OsO_4 in S-collidine for 1–2 hr at 0°. The samples were subsequently dehydrated in ethanol and embedded in Epon 812 for sectioning.

Statistical methods. Values are generally presented as means and standard deviations from three or four different experiments each involving livers pooled from several animals or individual animals.

Students *t*-test was used to evaluate statistical significance.

RESULTS

Effects of washing and increased centrifugal force on the purity of the subfractions

In order to get an idea of which differential centrifugation scheme to use before performing a detailed marker enzyme analysis, we tested the effects of washing on the membraneous fractions and of increased centrifugal force on the cytosolic fraction.

The mitochondrial fraction was essentially free of

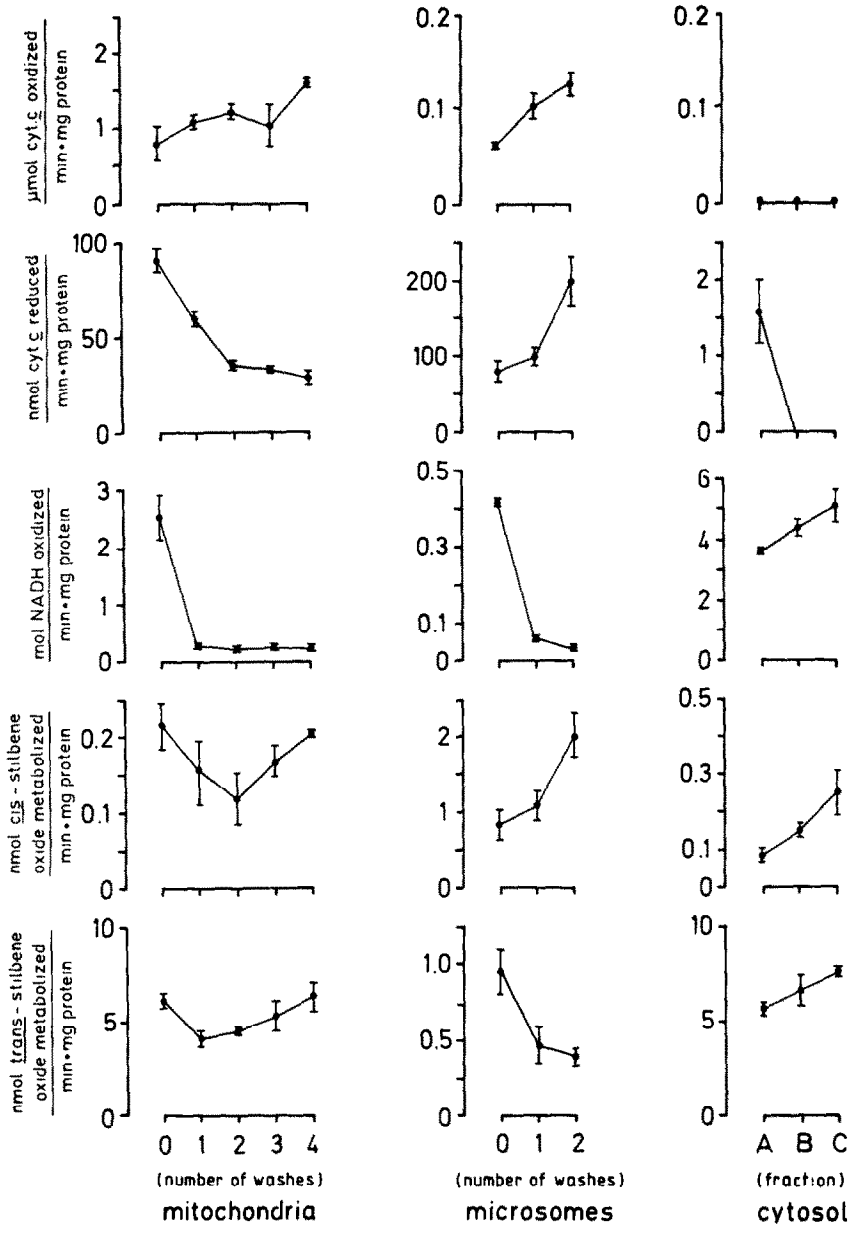

Fig. 1. Effects of washing or increased centrifugal force on the specific activities of some marker enzymes in the mitochondrial, microsomal and cytosolic fractions. The mitochondrial fraction (10,000 g_{av} pellet) was resuspended in 0.25 M sucrose and recentrifuged for 10 min at 10,000 g_{av}, while the microsomal fraction was resuspended in 0.15 M Tris–Cl, pH 8.0, and recentrifuged for 30 min at 105,000 g_{av}. The cytosolic fraction obtained after centrifugation for 60 min at 105,000 g_{av} (A) was further centrifuged for 60 min at 133,000 g_{av} (B) or for 120 min at 246,000 g_{av} (C). Enzyme activities (from top to bottom: cytochrome oxidase, NADPH-cytochrome *c* reductase, lactate dehydrogenase, epoxide hydrolase and epoxide hydrolase) were then measured as described in the Methods and the results expressed as means ± SD from three different experiments each involving livers pooled from five animals.

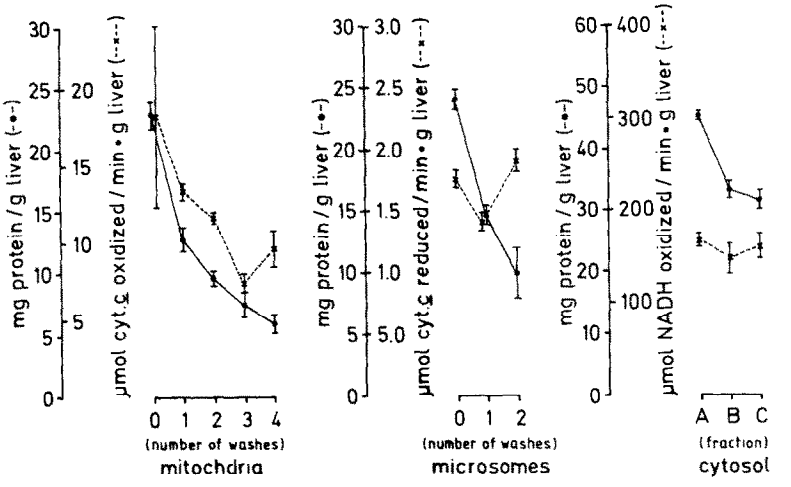

Fig. 2. Effects of washing or increased centrifugal force on the total recovery of major marker enzymes (from left to right: cytochrome oxidase, NADPH-cytochrome *c* reductase and lactate dehydrogenase) and protein in the mitochondrial, microsomal and cytosolic fractions. The conditions were the same as described in the legend to Fig. 1.

cytosolic contamination after one wash (as judged by lactate dehydrogenase activity). The specific cytochrome oxidase activity gradually increased with further washings, while the microsomal contamination (as judged by NADPH cytochrome *c* reductase activity) was minimized after two washes (Fig. 1). Specific epoxide hydrolase activity towards *trans*-stilbene oxide first decreased upon washing due to the removal of cytosolic protein, but then increased gradually as the mitochondria were further purified. A similar effect was also seen with *cis*-stilbene oxide as substrate, where the removal of microsomal protein caused the initial decrease in specific epoxide hydrolase activity.

Most of the contaminating cytosolic protein was removed from the microsomal fraction by one wash, whereas the cytochrome oxidase activity could not be washed away, as expected (Fig. 1). The NADPH-cytochrome *c* reductase activity increased significantly after the second wash, which may have been due to some effect of the Tris–chloride buffer on the membrane. In this case the specific epoxide hydrolase activity towards *cis*-stilbene oxide increased steadily with subsequent washes, while the reverse was true with *trans*-stilbene oxide as substrate.

The cytosolic fraction did not contain any intact mitochondria or inner mitochondrial membranes, while the NADPH-cytochrome *c* reductase activity was removed by using a higher sedimentation force than 105,000 g_{av} for 60 min (Fig. 1). The epoxide hydrolase activities increased somewhat with more extensive centrifugation.

Figure 2 illustrates the loss of total activities and protein in connection with these washings and increases in centrifugal force. In the case of the mitochondrial fraction washing caused a loss of both cytochrome oxidase activity and protein, probably due partially to fragmentation of mitochondria and the formation of submitochondrial particles. For the microsomal and the cytosolic fractions most of the major marker activity was retained, although there was a loss in total protein content.

A routine procedure for preparing relatively "pure" fractions with high recovery was then chosen. The mitochondrial fraction was washed twice and the microsomal fraction once and the supernatant obtained after the first 105,000 g_{av} centrifugation was centrifuged again at 133,000 g_{av} for 60 min to obtain a particle-free cytosolic fraction.

Subcellular distributions of different markers

Figure 3 shows the distributions of several markers after subcellular fractionation of mouse liver. DNA was used as a marker for nuclei, cytochrome oxidase for mitochondria, catalase for peroxisomes, *p*-nitrophenyl-α-mannosidase for the Golgi apparatus, β-glycerophosphatase for lysosomes, AMPase for the plasma membrane, glucose 6-phosphatase for the endoplasmic reticulum and lactate dehydrogenase for the cytosol. It is apparent today that few of these markers are localized to a single organelle in mammalian liver and, furthermore, that most are present either in the membrane or the soluble matrix of an organelle so that eventual disruption would

Fig. 3. deDuve plots for marker enzymes after subcellular fractionation of mouse liver. The subcellular fractions are, from left to right, the nuclear fraction (washed twice), the mitochondrial fraction (washed twice), the microsomal fraction (washed once) and the cytosolic fraction (B-fraction, Fig. 1). The specific activities for the markers in those fractions where they were most enriched were 70.0 ± 2.9 μg/mg protein (DNA), 1.47 ± 0.21 μmol cytochrome *c* oxidized/min · mg protein (cytochrome oxidase), 903 ± 222 μmol hydrogen peroxide oxidized/min · mg protein (catalase), 11.1 ± 0.86 nmol *p*-nitrophenyl-α-mannoside hydrolyzed/min · mg protein (*p*-nitrophenyl-α-α-mannosidase), 5.75 ± 0.09 nmol phosphate liberated/min · mg protein (β-glycerophosphatase), 35.3 ± 2.0 nmol phosphate liberated/min · mg protein (AMPase), 485 ± 46 nmol phosphate liberated/min · mg protein (glucose-6-phosphatase) and 338 ± 35 nmol NADH oxidized/min · mg protein (lactate dehydrogenase).

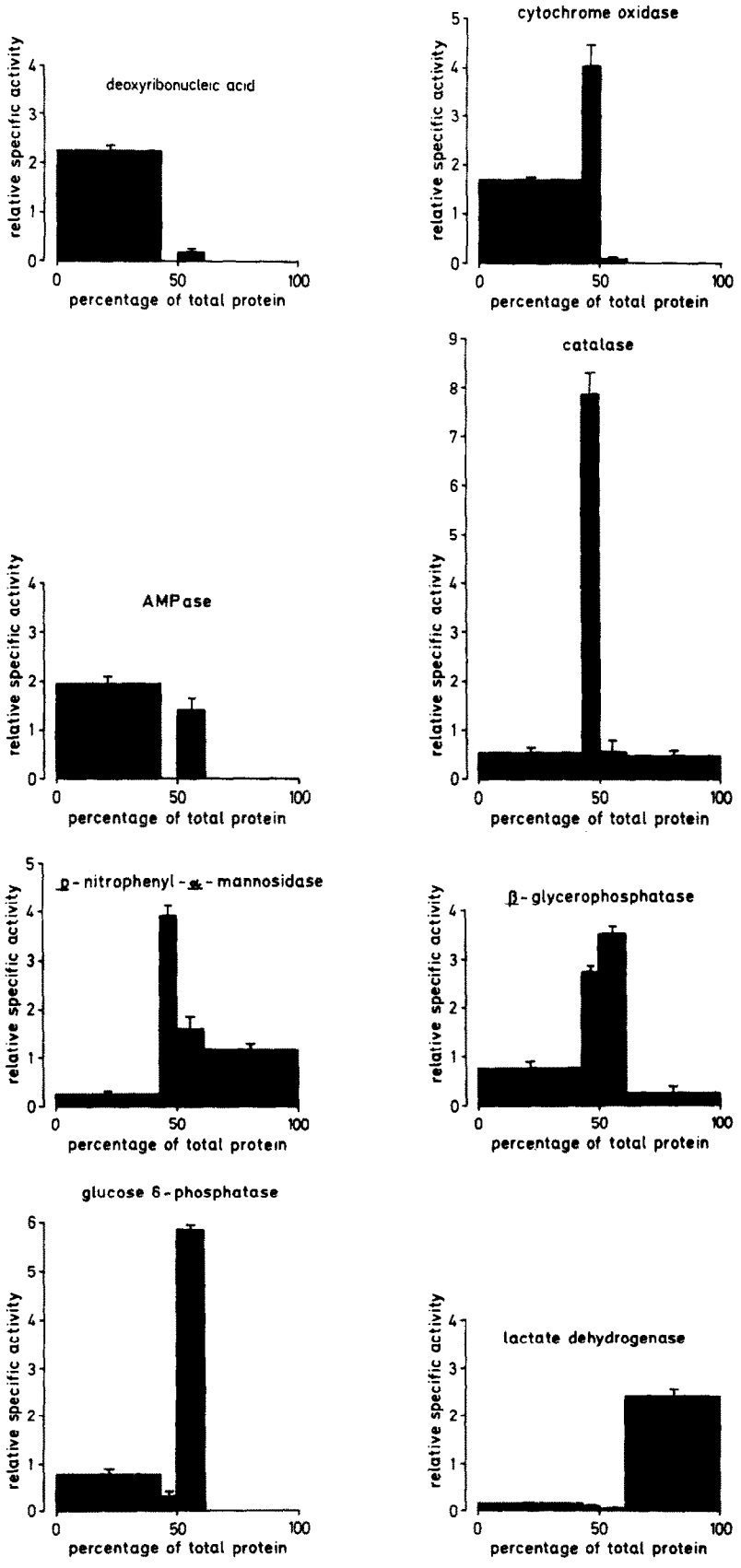
deoxyribonucleic acid
cytochrome oxidase
catalase
AMPase
p-nitrophenyl-α-mannosidase
β-glycerophosphatase
glucose 6-phosphatase
lactate dehydrogenase
relative specific activity
percentage of total protein
0
50
100

Fig. 3.

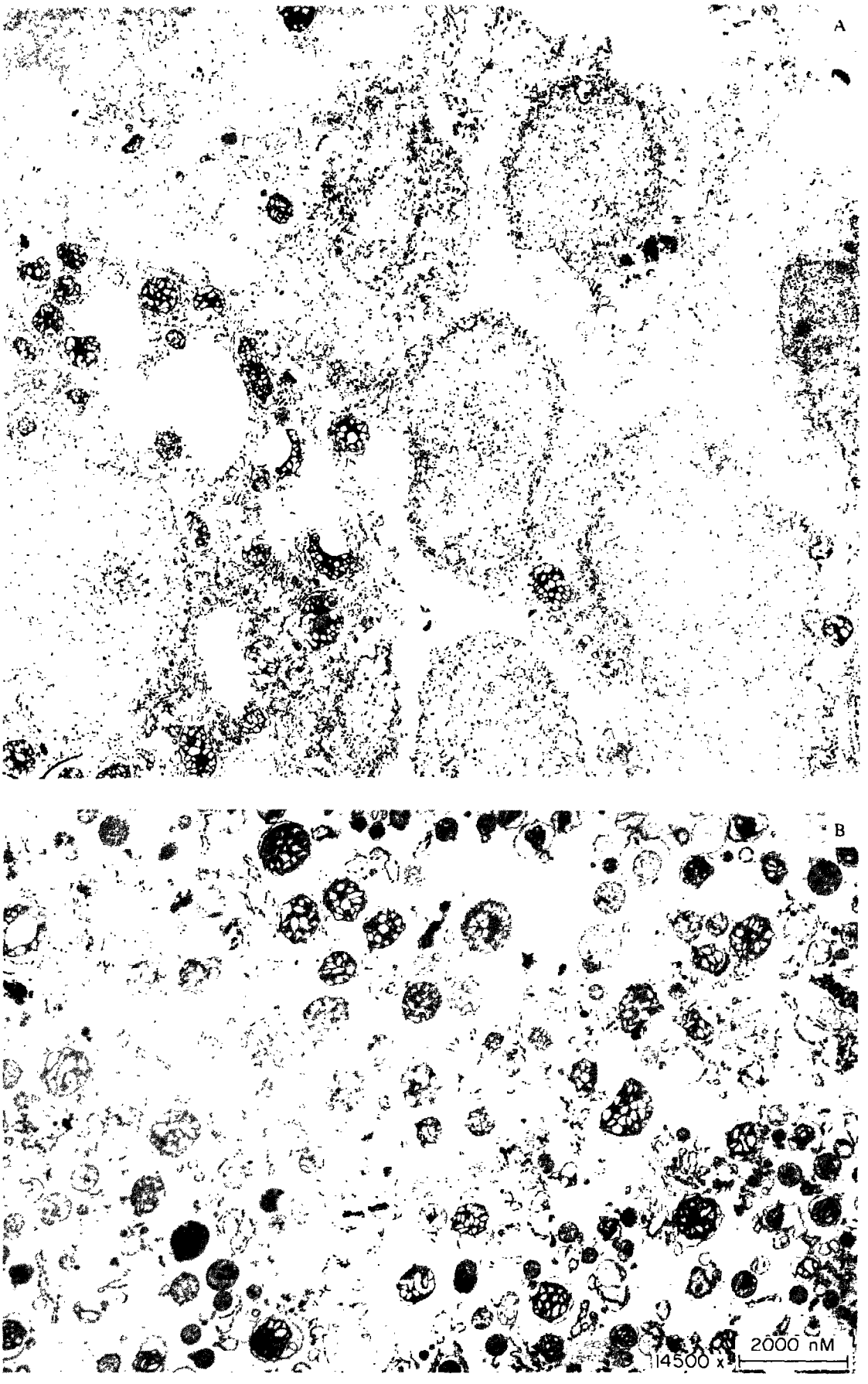

Fig. 4 (*continued*)

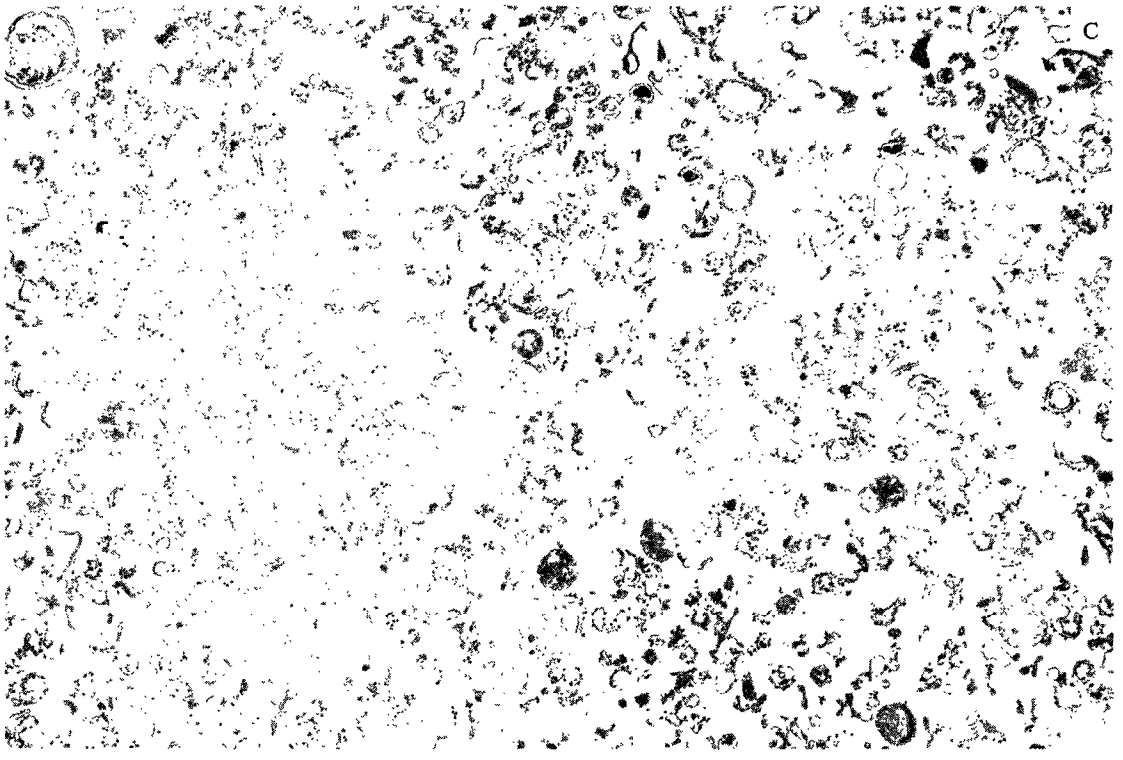

Fig. 4. Electron microscopy of the subcellular fractions. Fractions were prepared as described in the legend to Fig. 3 and Methods and pooled from four animals before fixation. The final magnifications are 9,500 × for the 600 g_{av} pellet (A), 9,000 × for the 10,000 g_{av} pellet (B) and 15,000 × for the 105,000 g_{av} pellet (C).

lead to an incorrect picture of the distribution of the whole organelle. Nonetheless, the larger part of each marker is localized to a single organelle and, if care is taken to avoid disruption, these markers provide a useful indication of the distribution of different subcellular structures.

The majority of the DNA (98%) was found in the nuclear fraction and the remaining in the microsomal, demonstrating good intactness of the nuclei (Fig. 3). The greatest enrichment of cytochrome oxidase was found in the mitochondrial pellet, although only 27% of the total activity was recovered in this fraction. The high level of cytochrome oxidase in the nuclear fraction (72%) was not simply due to undisrupted cells which would sediment at 600 g_{av}, since only 7% of the total lactate dehydrogenase activity was recovered in the nuclear fraction.

Catalase (peroxisomes) and *p*-nitrophenyl-α-mannosidase (Golgi apparatus) were enriched (53% and 26% of the total activity, respectively) in the 10,000 g_{av} pellet, as expected. The activities recovered in the cytosol are not surprising. Peroxisomes are quite fragile [30], thus allowing catalase to leak out, and catalase is also thought to occur normally in the cytosol [31]. α-Mannosidase activities have been found in lysosomes, the Golgi apparatus and in the cytosol of rat liver, but with different properties [27].

β-Glycerophosphatase activity (lysosomes) was, surprisingly, found to be most enriched in the 105,000 g_{av} pellet, which contained 40% of the total activity, while only 18% was recovered in the 10,000 g_{av} pellet. Whether this is due to the presence of this enzyme activity in the endoplasmic reticulum in this tissue or reflects solely contamination by lysosomes remains to be established. Rat liver microsomes normally contain about 10% of the total lysosomes [32] and this figure may be even higher in mouse liver.

AMPase was distributed bimodally between the nuclear and microsomal fractions (84% and 16% of the total activity, respectively), reflecting the disruption of this structure into both large sheets and small vesicles upon homogenization. A 6-fold enrichment (67% of the total activity) of glucose-6-phosphatase was found in the 105,000 g_{av} pellet. 92% of the lactate dehydrogenase activity was found in the cytosol, indicating effective disruption of the hepatocytes.

Electron microscopy of the subcellular fractions

The purity of the subcellular fractions was also analyzed using electron microscopy. The 600 g_{av}, 10,000 g_{av} and 105,000 g_{av} pellets were analyzed, while the 133,000 g_{av} supernatant did not contain any membraneous structures that could be sedimented after fixation.

As can be seen in Fig. 4, the 600 g_{av} pellet contained some mitochondria in addition to the nuclei. The 10,000 g_{av} pellet was highly enriched in mitochondria in the condensed state, which is the normal case [33]. In addition, this fraction contained peroxisomes, lysosomes and rough and smooth vesicles, some of which were probably derived from the endo-

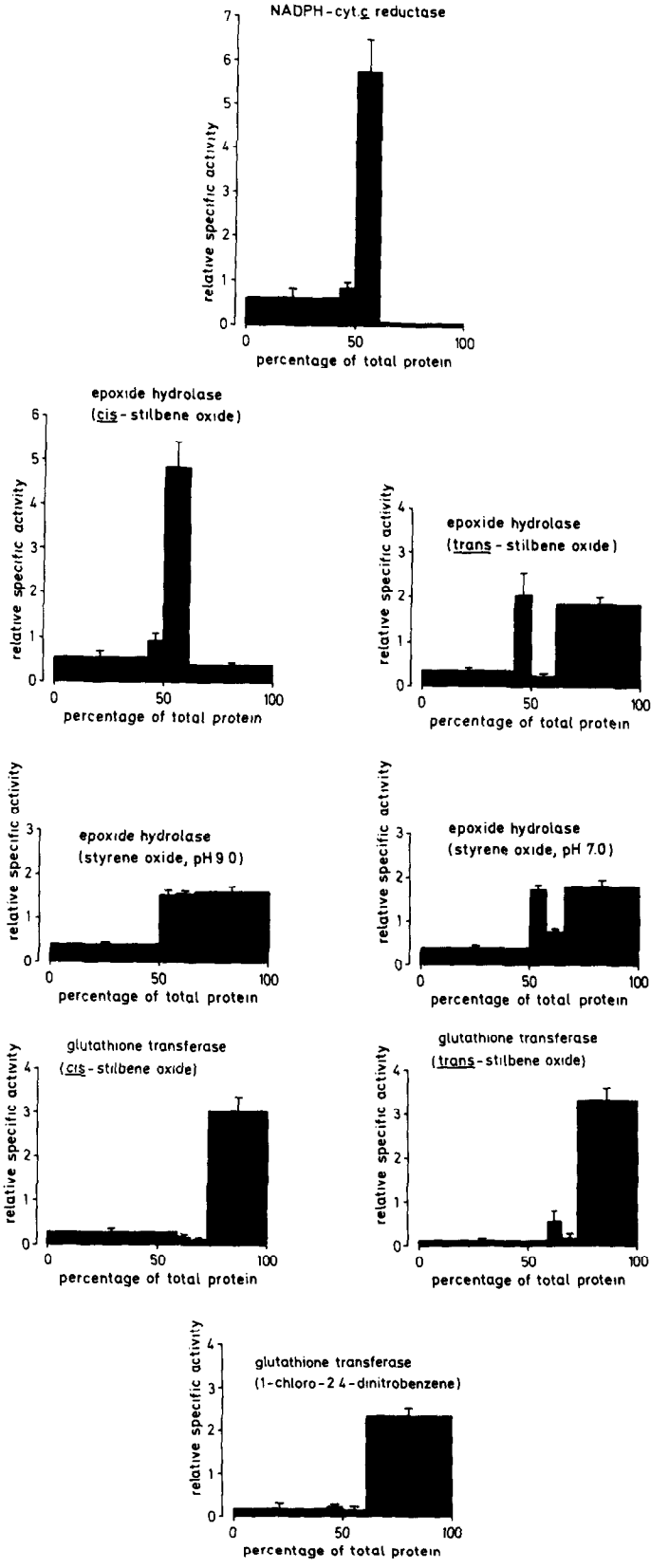

Fig. 5. deDuve plots for certain xenobiotic-metabolizing enzymes after subcellular fractionation of mouse liver. Fractions were prepared as described in the legend to Fig. 3 and Methods. The specific activities for the enzymes in those fractions where they were most enriched were 23.4 ± 2.4 nmol cytochrome *c* reduced/min·mg protein (NADPH-cytochrome *c* reductase), 1.50 ± 0.10 and 3.32 ± 0.98 nmol stilbenediol formed/min·mg protein (epoxide hydrolase activity towards *cis*- and *trans*-

plasmic reticulum. The 105,000 g_{av} pellet contained almost exclusively small rough and smooth vesicles without any other typical organelle structures.

Subcellular distributions of certain xenobiotic-metabolizing enzymes

We also determined the distributions of several enzymes of xenobiotic metabolism in our subfractions. The profile of NADPH-cytochrome *c* reductase confirmed the purity and high recovery (66%) of endoplasmic reticulum in the microsomal fraction (Fig. 5).

Epoxide hydrolase activity with *cis*-stilbene oxide as substrate also showed the highest (5-fold, 55% of the total activity) enrichment in the microsomal fraction. In this case we know that purified epoxide hydrolase from mouse liver cytosol [34] and mitochondria (unpublished observations) also demonstrates low activity towards *cis*-stilbene oxide. With *trans*-stilbene oxide as substrate, a bimodal distribution of epoxide hydrolase activity in the mitochondrial and cytosolic fractions (with 14 and 70% of the total activity, respectively) was obtained. On the other hand, with styrene oxide as substrate at pH 9 similar enrichments in the mitochondrial, microsomal and cytosolic fractions were observed. When the assay was performed with this latter substrate at pH 7 instead, the mitochondrial and cytosolic fractions had the highest specific activities, while the latter fraction had the highest total activity at both pHs (about 60%).

Glutathione transferase activity with 1-chloro-2,4-dinitrobenzene, *cis*- and *trans*-stilbene oxide as second substrates was distributed in a manner very similar to lactate dehydrogenase. No activation with *N*-ethylmaleimide was performed in order to study specifically microsomal glutathione transferase activity, but this enzyme is present and can be activated in mouse liver as well [35].

Effects of starvation on some xenobiotic-metabolizing and marker enzymes

Since dietary status may affect xenobiotic metabolism and since starvation is routinely used to reduce hepatic glycogen before subfractionation, we starved mice for 1 or 2 days (3 days of starvation was found to have toxic or lethal effects) and compared the resulting epoxide hydrolase activities in the different subcellular fractions (Fig. 6). With *cis*-stilbene oxide as substrate, significant decreases in specific activity occurred in the nuclear, mitochondrial and microsomal fractions after 2 days of starvation and also after 1 day in the mitochondrial fraction. With *trans*-stilbene oxide as substrate, small changes in activity were observed. The activity in the nuclear fraction decreased after starvation, while the activities increased in the mitochondrial and cytosolic fractions after 2 days of starvation. After starvation for 1 day, the same deDuve plots as for non-starved animals with respect to cytochrome oxidase, catalase, NADPH-cytochrome *c* reductase, epoxide hydolase (*cis*- and *trans*-stilbene oxide and styrene oxide), lactate dehydrogenase and glutathione transferase (1-chloro-2,4-dinitrobenzene) were obtained (results not shown). Apparently, starvation does not affect the enzymes measured to any great extent.

Effect of storage at 4° and 20° and of freeze-thawing on epoxide hydrolase activities

In order to investigate the stability of the different epoxide hydrolase activities, we stored subcellular fractions at 4° or 20° with or without nitrogen flushing and assayed for activity at different time points. All fractions were found to retain a high and rather constant activity for the first hours after preparation when stored at 4° (Fig. 7) or at 20° (not shown) under a nitrogen atmosphere. The activity then slowly declined, but activity was still measurable after one week. A nitrogen atmosphere did not seem to have a marked protective effect on the activity.

The epoxide hydrolase activities were remarkably stable after several rounds of freeze-thawing (Table 1). No large effects were seen after one freeze-thawing for any fraction and after 6 freeze-thawings the activities towards *cis*-stilbene oxide and, to a lesser extent, the cytosolic activity towards *trans*-stilbene oxide had decreased significantly. Furthermore, these enzyme activities are stable for at least 3 years when stored at −20° or −70°.

Lack of effect of rat liver cytosol and mouse bile on epoxide hydrolase activity

Since rat liver demonstrates very low mitochondrial and cytosolic epoxide hydrolase activities compared to several other mammalian species, we performed several experiments to exclude certain possible explanations for this difference. One obvious possibility is that rat liver contains an inhibitor for the cytosolic and mitochondrial forms. The cytosolic form is known to be very sensitive to inhibition by several small compounds, e.g. naphthoflavones, benzil and chalcone oxides [34, 36]. We therefore preincubated rat liver cytosol with mouse liver cytosol and mitochondria before performing the assay. No significant differences were observed in this experiment (Table 2). One possible explanation for the lack of an inhibitory effect of rat liver cytosol on mouse liver epoxide hydrolase could be that the potential inhibitor is present at such a low concentration that it is already mostly bound to the rat liver enzyme and cannot give rise to further inhibition.

A possible site of inhibitory substances of epoxide hydrolase in the liver is the bile. Diol metabolites, possible competitive inhibitors, can be excreted in the bile; bile acids are strong detergents which can disturb the structure of enzymes and are present at high concentrations; and excretion products like

stilbene oxide, respectively), 3.45 ± 0.20 and 1.63 ± 0.12 nmol styrene diol formed/min·mg protein at pH 7 and pH 9, respectively (epoxide hydrolase with styrene oxide as substrate), 1.87 ± 0.38 μmol 1-chloro-2,4-dinitrobenzene conjugated/min·mg protein (glutathione transferase) and 49.8 ± 5.0 and 5.80 ± 0.90 nmol *cis*-and *trans*-stilbene oxide conjugated/min·mg protein, respectively (glutathione transferase).

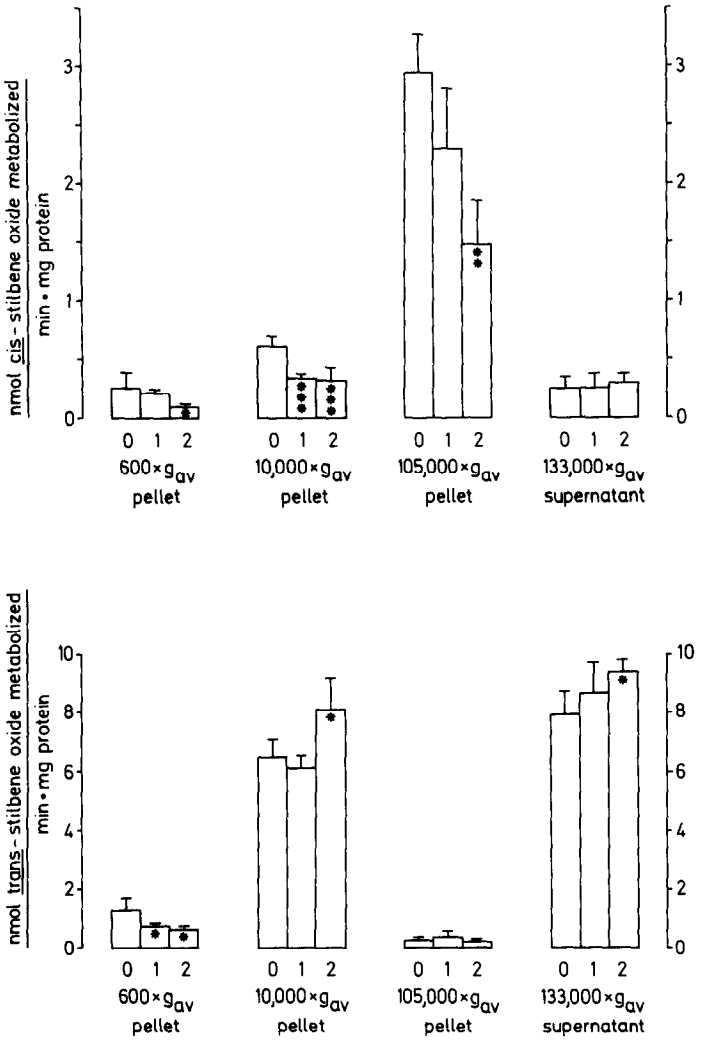

Fig. 6. Effects of starvation on epoxide hydrolase activities in mouse liver. Mice with free access to food (0) or starved for 1 or 2 days were used and fractions prepared as in the legend to Fig. 3. Epoxide hydrolase activities were measured as described in the Methods and the results are expressed as means ± SD for four animals.

Table 1. Effects of freeze-thawing on epoxide hydrolase activities in different subcellular fractions from mouse liver

Fraction	Substrate	No. of freeze-thawings	% of control activity
Mitochondrial	*cis*-stilbene oxide	1	85.1 ± 7.0
		6	64.5 ± 8.2†
	trans-stilbene oxide	1	101.5 ± 10.4
		6	103.4 ± 14.1
Microsomes	*cis*-stilbene oxide	1	94.2 ± 13.6
		6	72.7 ± 5.5†
Cytosol	*trans*-stilbene oxide	1	87.9 ± 1.1†
		6	91.6 ± 2.6*

The results are presented as means ± SD from four mice treated individually. At least 24 hr were allowed to pass between subsequent freeze-thawings and the fractions were flushed with nitrogen before freezing again. Statistical significance was determined using Student's *t*-test. * $P < 0.05$; † $P < 0.01$.

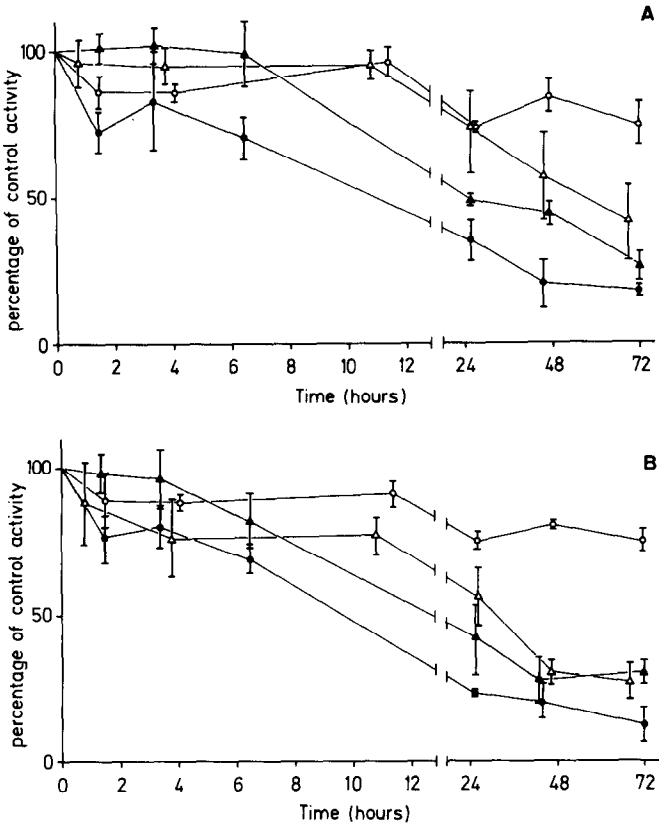

Fig. 7. Effect of storage time on epoxide hydrolase activities in different subcellular fractions from mouse liver. Fractions were prepared as described in the legend to Fig. 3 and immediately assayed for epoxide hydrolase activity [14]. Aliquots were stored on ice in 0.25 M sucrose with (A) or without (B) nitrogen atmosphere and assayed at different time points. Results are expressed as means ± SD for 4 animals. The fractions assayed were the mitochondrial fraction with *cis*-(–▲–) and *trans*-stilbene oxide (–●–), the microsomal fraction with *cis*-stilbene oxide (–△–) and the cytosolic fraction with *trans*-stilbene oxide (–○–).

bilirubin which binds, for example, to glutathione transferase [37] and other potential inhibitory compounds are present in the bile. As a precautionary measure, however, we routinely remove the gallbladder before handling the mouse liver further.

Table 2. Lack of effect of rat liver cytosol and mouse bile on epoxide hydrolase activities towards *trans*-stilbene oxide in different subcellular fractions from mouse liver

Fraction	Addition	% of control activity
Mitochondria	1% rat liver cytosol	80.4 ± 6.9
	0.01% bile	85.9 ± 7.6
	1% bile	124.4 ± 6.6*
Cytosol	1% rat liver cytosol	87.6 ± 10.7
	0.01% bile	78.6 ± 5.1
	1% bile	87.7 ± 8.1

The results are presented as means ± SD for four mice treated individually. The vials were preincubated for 10 min with rat liver cytosol pooled from two rats or with bile collected from gallbladders of unstarved mice. The bile itself was not found to affect the partitioning of the epoxide or the diol in the assay procedure. Statistical significance was determined using Student's *t*-test. * $P < 0.05$.

The rat lacks a distinct gallbladder and the contents of the bile cannuliculi are mixed with the liver cells during the homogenization procedure. If there is an inhibitor in this bile, the low level of epoxide hydrolase activity in rat liver cytosol would then be an *in vitro* artefact.

In order to look for such an inhibitor we took bile from the gallbladders of starved mice and preincubated it with mouse liver mitochondria and cytosol (Table 2). In this experiment as well, no marked inhibition was observed and, indeed, in the case of mitochondria the activity actually increased with 1% bile.

DISCUSSION

Due to the lack of published data concerning subfractionation of mouse liver, we felt it important to do a limited characterization of some easily obtainable fractions. It seemed reasonable to begin by using conditions which have been developed for the subfractionation of rat liver and modify this procedure.

We think it is very important to know the composition and purity of the routinely used mitochondrial, microsomal and cytosolic fractions from

mouse liver for several reasons. It is now apparent that epoxide hydrolase activities can be found in all three of these fractions in mouse liver [11, 15] and several other species as well (J. Meijer, G. Lundqvist and J. W. DePierre, manuscript in preparation). It is therefore important to know if the activities found in the different fractions are due to the main component in the fraction isolated or due to contamination of other organelles recovered in this fraction as well. For instance, there are speculations that there may exist a new form of epoxide hydrolase in rat liver microsomes which has very similar properties to cytosolic epoxide hydrolase [38]. For enzyme purification it is also important to have pure fractions to decrease the risk of heterogeneity in the final preparation. Such fractions should also be useful in other respects, e.g. in studies concerning the biogenesis and transport of newly synthesized proteins and for elucidation of the relative contribution of different subcellular fractions and isozymes to the metabolism of different compounds.

It is apparent from the present study that reasonably pure fractions can be obtained from mouse liver by a procedure analogous to that used for rat (keeping in mind the limitations of marker enzyme analysis). Two washes for the mitochondrial fraction, one wash for the microsomal fraction and an increased gravitational force on the cytosolic fraction resulted in maximal and constant specific activities of the major marker enzymes. The only major difference as compared to rat liver was the high β-glycerophosphatase activity recovered in the microsomal fraction.

Epoxide hydrolase activities were found in all fractions studied, as expected. Our results confirm earlier findings that epoxide hydrolase activity towards *trans*-stilbene oxide is enriched in both the "mitochondrial" and cytosolic fractions from mouse liver, i.e. exhibits bimodal distribution upon subcellular fractionation [39, 40]. Concerning the "mitochondrial" activity recent studies have shown this activity to be localized to peroxisomes [41, 42] or to mitochondria and peroxisomes from mouse liver [43].

We also find that *cis*- and *trans*-stilbene oxide are selective substrates for the microsomal and cytosolic/"mitochondrial" forms of epoxide hydrolase, respectively, confirming earlier reports [15]. On the basis of these and other findings [14], we suggest that epoxide hydrolase activity towards *cis*-stilbene oxide may be an excellent marker for the endoplasmic reticulum. Most or all of this activity in the "mitochondrial" fraction could be accounted for by cross-contamination with microsomes. Styrene oxide is about equally well metabolized by all the different isozymes of epoxide hydrolase in mouse liver at pH 9, but is somewhat selective for the cytosolic/"mitochondrial" form at pH 7.

Glutathione transferase activities towards *cis*- and *trans*-stilbene oxide and 1-chloro-2,4-dinitrobenzene were all most highly enriched in the cytosolic fraction, as also expected. However, there was relatively somewhat more glutathione transferase activity with *trans*-stilbene oxide than with the other two substrates in the "mitochondrial" fraction, suggesting that the isozymes of glutathione transferase known to be present in mitochondria [44] may conjugate *trans*-stilbene oxide especially effectively. A calculation of the relative contribution of glutathione transferases and epoxide hydrolases in the mitochondrial, microsomal and cytosolic fractions to the total metabolism of *cis*- and *trans*-stilbene oxide was performed. The total turnovers of both substrates in the different cell compartments were nearly equal. This calculation assumes, of course, that optimal activities measured *in vitro* are applicable to the intact cell. Glutathione transferases would be expected to metabolize 89% of *cis*-stilbene oxide *in vivo* (where the cytosol contributes 89% of this activity) and the remaining 11% is metabolized by epoxide hydrolases (where microsomes catalyze 52% of the conversion). *trans*-Stilbene oxide, on the other hand, is more extensively metabolized by epoxide hydrolases (64%) than glutathione transferases (36%). For this substrate the cytosol is the major metabolizing compartment, containing 75 and 88% of the total activities of epoxide hydrolases and glutathione transferases, respectively.

The epoxide hydrolase activities in the different fractions were found to be surprisingly stable. Several rounds of freeze–thawing do not affect these activities to any great extent and storage for several years in a freezer does not affect the activity significantly. The activities are also quite constant after preparation when stored at 4° for the first day and it is, therefore, not necessary to assay fractions immediately after preparation.

Starvation did not affect the distribution of the marker enzymes studied and had only minor effects on the different epoxide hydrolase activities. Accordingly, starvation can be used routinely in such studies to reduce the glycogen content of the liver.

We did not find any inhibitory effect of rat liver cytosol or mouse bile on mouse liver epoxide hydrolase activities. Thus, there would appear to be no or very low levels of an endogenous inhibitor of cytosolic epoxide hydrolase in rat liver. The difference in activity between rat and mouse liver thus seems to reflect a difference in protein content, which is also confirmed by immuno-chemical quantitation using antibodies towards purified mouse liver cytosolic epoxide hydrolase (unpublished).

We are presently investigating the distribution of different forms of epoxide hydrolases in the different subcellular fractions with immunochemical procedures.

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