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STUDIES ON LYSOPHOSPHOLIPASES

IV. THE SUBCELLULAR DISTRIBUTION OF TWO LYSOLECITHIN-HYDROLYZING ENZYMES IN BEEF LIVER

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Summary

1. In a previous paper (Biochim. Biophys. Acta (1974) 369, 50—63) the purification of two proteins with lysophospholipase activity (EC 3.1.1.5), provisionally denoted lysophospholipase I and lysophospholipase II, has been described. The subcellular localization of both enzymes was investigated by cell fractionation studies.

2. For each subcellular fraction the total lysophospholipase activity, after solubilization by *n*-butanol treatment, was separated into a lysophospholipase I and II contribution by DEAE-Sephadex ion exchange chromatography.

3. Lysophospholipase I was found to be a soluble enzyme with a bimodal distribution. Highest relative specific activities were measured in the mitochondrial and the cytoplasmic fraction. Evidence is presented indicating that this enzyme is present in the mitochondrial matrix fraction.

4. Lysophospholipase II appeared to be a membrane-bound enzyme with highest relative specific activity in the microsomal fraction.

Introduction

The presence of lysophospholipase activities has been reported for various subcellular fractions of many tissues (for a review, see ref. 1). However, only a few studies have concentrated on the quantitative aspects of the subcellular distribution of the lysophospholipase activity in relation to the purity of the isolated subcellular fractions. Bjørnstad, when studying the phospholipase activities in rat liver mitochondria [2] and microsomes [3] using endogenous substrates, noticed that the initially formed lysophosphoglycerides were con-

verted into the completely deacylated phosphodiesters to a much higher extent by microsomes than by mitochondria. Erbland and Marinetti [4] and van den Bosch et al. [5] concluded from their studies using exogenous lysophosphoglycerides that the bulk of the lysophospholipase activity was present in the 100 000 × *g* supernatant. A similar distribution was reported for the enzyme of rat spleen [6]. Hörtnagl et al. [7] found most, if not all, of the lysophospholipase activity in bovine adrenal medulla to be localized in the microsomal fraction. Leibovitz and Gatt [8] reported the microsomal fraction of rat brain to have the highest specific lysophospholipase activity, but found lysophospholipases in the mitochondrial and cytosol fraction as well. The particulate and soluble lysophospholipase exhibited quite different kinetic properties [9].

In a previous paper [10] we have reported on the purification of two lysophospolipases, provisionally designated lysophospholipase I and II, with quite different molecular weights and isoelectric points, from beef liver. In view of the scarce knowledge about the subcellular localization of lysophospholipases and the fact that different enzymes may contribute to the total tissue lysophospholipase activity we have determined the subcellular distribution of both lysophospholipase I and lysophospholipase II. In addition, the possible relationship between both enzymes was investigated.

Materials and Methods

Cellfractionation

Fresh beef liver was obtained from the local slaughterhouse. A 10%-homogenate in 0.25 M sucrose containing 5 mM Tris-HCl buffer, pH 7.2 and 2 mM EDTA was prepared from 50 g of beef liver with the use of a Potter-Elvehjem tube. This homogenate was filtered through one layer of cheese cloth and then subjected to the classical centrifugation procedure to prepare subcellular fractions from liver homogenates. Centrifugation was done as follows; 5 min at 1000 × *g*, 10 min at 9000 × *g*, 10 min at 20 000 × *g* and 60 min at 100 000 × *g* to yield pellets enriched in nuclei and debris, mitochondria, lysosomes and microsomes as well as a cytosol fraction. Pellets were resuspended in known volumes of 20 mM Tris-HCl buffer, pH 7.2, in 0.125 M KCl. Aliquots of 1 ml of these fractions were frozen at −16°C and thawed only once for determination of enzymatic activities. Likewise, larger quantities were frozen and thawed only once for the preparation of delipidated extracts to be used for the separation of lysophospholipase I and lysophospholipase II.

For the preparation of purified mitochondria the crude 9000 × *g* pellet was washed once with the sucrose/Tris/EDTA solution and resuspended in this mixture. Of this suspension 4 ml, corresponding to 4 g of beef liver, was loaded on top of a discontinuous sucrose gradient ranging from 20 to 55% sucrose as described by Sarzala et al. [11]. The tubes were then centrifuged during 30 min at 25 000 rev./min in a SW 27 rotor in a Beckman L2-65B centrifuge. Mitochondria were harvested with the use of a bent needle attached to a syringe. In some experiments the crude lysosomal fraction (20 000 × *g* pellet) was purified over the same gradient.

Enzyme assays

The purity of the subcellular or submitochondrial fractions was estimated

by assaying the fractions for marker enzyme activities. Glucose-6-phosphatase (EC 3.1.3.9) and rotenone-insensitive NADPH-cytochrome *c* reductase (EC 1.6.2.3.) were used as microsomal marker enzymes and were assayed as described in refs 12 and 13. Acid phosphatase, (EC 3.1.3.2.) as a lysosomal marker, was assayed with β-glycerophosphate as substrate according to Gianetto and de Duve [14]. Succinate dehydrogenase (EC 1.3.9.1), assayed according to Green et al. [15], served as a marker for mitochondria and inner mitochondrial membranes. Malate dehydrogenase (EC 1.1.1.27) was determined as described by Beaufay et al. [16] and used as a marker for the mitochondrial matrix fraction. Alternatively, glutamate dehydrogenase (EC 1.4.1.2), assayed according to Scholte [17] was used as marker for the mitochondrial matrix. For the interstitial soluble protein fraction adenylate kinase (EC 2.7.4.3) was used as marker and assayed as described by Sottacasa [18]. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was used as cytosol marker and assayed according to Bergmeyer et al. [19]. Lysophospholipase activity (EC 3.1.1.5) was determined as described previously [20] with synthetic 1-[1-^{14}C]palmitoyl lysolecithin as substrate. The incubation mixtures for both lysophospholipase I and lysophospholipase II contained 0.4 mM substrate and 2 mM β-mercaptoethanol in 0.5 ml of 20 mM potassium phosphate buffer (pH 7.5).

Separation of lysophospholipases I and II

For the separation of lysophospholipase I and II in a particular subcellular fraction an aliquot of this fraction (about 400 mg protein) was stirred for 5 min at 0°C with an equal volume of *n*-butanol saturated with water. After centrifugation for 10 min at 15 000 rev./min in the SS 34 rotor of a Sorvall RC2B the upper butanol layer was removed with a Pasteur pipet connected to an aspirator. The infranatant was decanted through a funnel with a piece of cotton wool into dialysis tubing. The lipid-free extract was then dialysed overnight against 50 vol. 20 mM Tris-HCl buffer (pH 7.3) in 100 mM NaCl and, if necessary, briefly centrifuged to yield a clear extract. This solution was applied to a small DEAE-Sephadex column (10 cm × 1.5 cm) equilibrated with the dialysis buffer. The lysophospholipase I was eluted in the breakthrough peak. After elution of this peak the column was eluted with a linear gradient of 100 ml each of 100 mM and 300 mM NaCl in 20 mM Tris-HCl buffer (pH 7.3). The lysophospholipase II was eluted at 150—250 mM NaCl. Fractions of 9 ml were collected at a flow rate of 18 ml/h.

Alternatively, when the ratio of lysophospholipase I to II were to be determined in gradient fractions, the lipid-free extracts were applied to a mini DEAE-Sephadex column in a Pasteur pipet (bed volume 0.5 ml). Control experiments indicated that enzyme I was quantitatively eluted with 4 ml of the 100 mM NaCl solution. Lysophospholipase II was then collected by elution with 4 ml of 400 mM NaCl in the Tris buffer, pH 7.3.

Analytical methods

Protein was determined according to Lowry et al. [21]. Spectrophotometric assays of marker enzymes were done with an Unicam SP 500 equipped with a recorder.

Results

The distribution of protein and marker enzymes over the various subcellular fractions obtained during fractionation of beef liver is indicated in Fig. 1. In general a very good recovery of enzymic activity after fractionation was obtained.

The relative specific activities of the marker enzymes in the subcellular fractions were calculated and plotted as recommended by de Duve et al. [22] (Fig. 1). Clearly, succinate dehydrogenase was found to have its highest relative specific activity in the mitochondrial fraction. Glucose-6-phosphatase showed a typical microsomal distribution. Glucose-6-phosphate dehydrogenase was recovered almost completely in the cytosol fraction. The lysosomal marker, acid phosphatase, quite unexpectedly showed its highest relative specific activity in the 100 000 × *g* pellet, rather than in the 20 000 × *g* pellet, as it does in rat liver [23]. A similar distribution as given in Fig. 1 was found repeatedly and was also obtained when the acid phosphatase activity was assayed in the presence of 0.1% Triton-X100. Either beef liver contains more enzymes capable of releasing inorganic phosphate from glycero-2-phosphate at acid pH values or beef liver lysosomes require higher *g*-forces than those from rat liver to be spun down. It is quite possible that this difference is related to the nutritional state of the liver as Tulkens and Wattiaux [24] have shown that the lysosomal fraction contained a smaller percentage and the microsomal fraction a higher percentage of the total acid phosphatase activity of the homogenate when fed rats were compared with fasted animals.

The total lysophospholipase activity follows more or less the microsomal

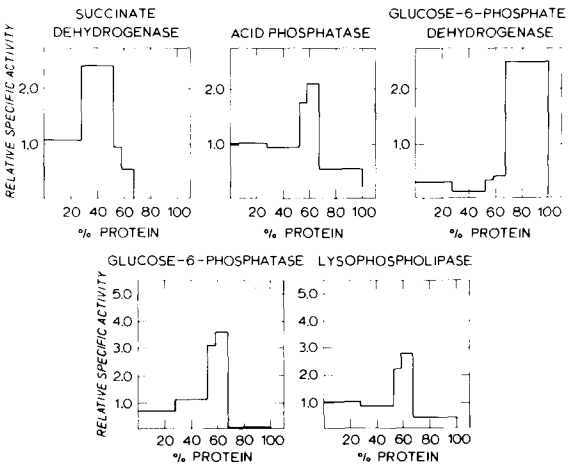

Fig. 1. Relative specific activities of lysophospholipase and marker enzymes versus percentage of total recovered protein in subcellular fractions from beef liver. From left to right: debris and nuclear, mitochondrial, lysosomal, microsomal and cytosol fraction. Enzymic activities in the total homogenate, expressed in μmol substrate converted per min, and total recoveries thereof in subcellular fractions, expressed as percent of homogenate activity, were as follows: Succinate dehydrogenase, 28.00, recovery 106%; Acid phosphatase, 32.60, recovery 96%; Glucose-6-phosphate dehydrogenase, 16.10, recovery 86%; Glucose-6-phosphatase, 375, recovery 102%; Lysophospholipase, 25.50, recovery 81%. The total homogenate contained 7650 mg protein, recovery 100%.

distribution observed for glucose-6-phosphatase, although in comparison with this marker there is clearly more lysophospholipase activity in the cytosol fraction. However, the total lysophospholipase activity measured in the subcellular fraction is the sum of probably quite varying ratios of lysophospholipase I and lysophospholipase II. Therefore, the lysophospholipase activity from each fraction was solubilized by *n*-butanol treatment and separated into lysophospholipase I and II as described under Materials and Methods. The results of these experiments are represented in Table I and Fig. 2. After treatment of the subcellular fractions with *n*-butanol to solubilize the lysophospholipases and dialysis of the water layer overnight to remove the butanol about 75—85% of the total lysophospholipase activity was recovered in the lipid-free extract. After DEAE-Sephadex chromatography the sum of the recovered lysophospholipase I and II activities amounted from 77% up to 99% of the total lysophospholipase activity applied to the column (Table I).

The microsomal fraction contained essentially only lysophospholipase II, whereas the lysophospholipase activity in the cytosol fraction is mainly due to lysophospholipase I (Table I and Fig. 2). The lysosomal fraction, i.e. the 20 000 × *g* pellet, also appears to have almost exclusively lysophospholipase II activity. Although the microsomal fraction at one hand and the cytosol fraction on the other hand contain largely different lysophospholipase the total recovery of enzymic activity after solubilization and ion-exchange chromatography is 74% for the microsomes and 76% for the cytosol. Thus, within the accuracy of the determination, the ratio of lysophospholipase I and II in a given subcellular fraction can be assumed to be equal to this ratio found in the DEAE-column eluate from that fraction. By calculation it is then possible to separate the total lysophospholipase activity of a fraction into a lysophospholipase I and a lysophospholipase II contribution (Table II).

TABLE I

SOLUBILIZATION AND SEPARATION OF LYSOPHOSPHOLIPASES FROM SUBCELLULAR FRACTIONS

The amounts of protein and lysophospholipase activity used for each subcellular fraction are indicated in Columns 1 and 2. The lysophospholipase activity recovered in the lipid-free extract after *n*-butanol treatment is given in m units in Column 3 and as a percentage of the initial activity in Column 4. These extracts were then chromatographed over DEAE-Sephadex to separate lysophospholipase I and II. Columns 5 and 7 sum up the lysophospholipase I and lysophospholipase II activity, respectively, whereas the recovery of activity after ion exchange chromatography is indicated in Column 9. The proportional distribution of lysophospholipase I and II (as percent of total recovered activity) is represented in Columns 6 and 8, respectively.

Fraction	Starting material		Lipid-free extract		Lysophospholipase I		Lysophospholipase II		Total recovery
	(mg protein) (1)	(m units) (2)	(m units) (3)	(%) (4)	(m units) (5)	(%) (6)	(m units) (7)	(%) (8)	(%) (9)
Nuclei, debris	390	1055	835	79	143	22	500	78	77
Mitochondria	437	1000	730	73	211	38	352	62	77
Lysosomes	353	2118	1655	78	52	4	1438	96	90
Microsomes	444	3330	2496	75	38	2	2425	98	99
Cytosol	427	451	383	85	304	89	37	11	89

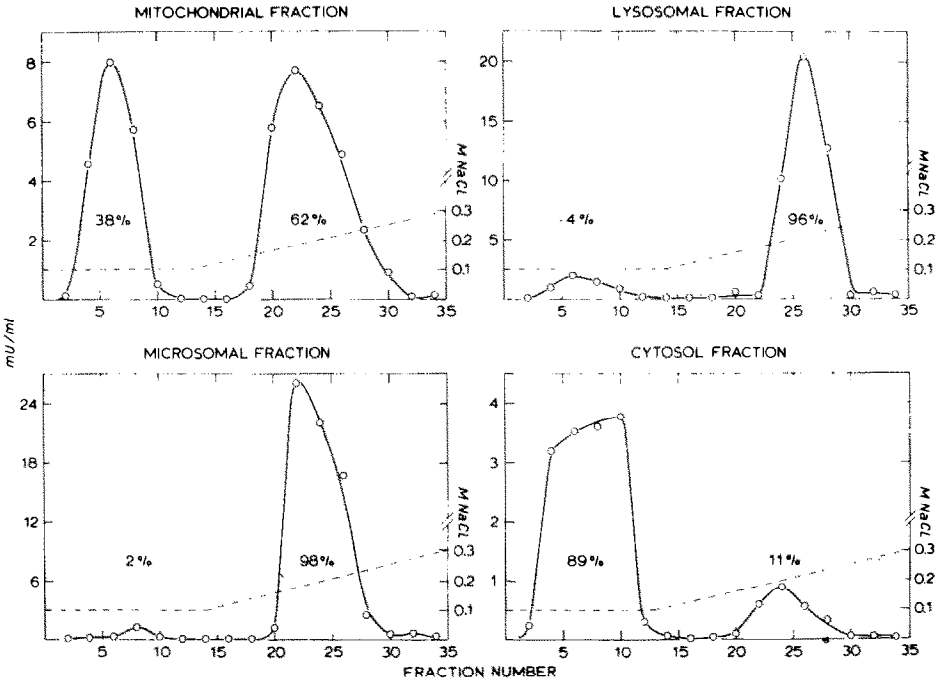

Fig. 2. Separation of lysophospholipase I and lysophospholipase II from various subcellular fractions. For experimental details see also Materials and Methods and Table I. For simplicity the protein contents of the fractions have not been plotted. Of the indicated fractions 0.2 ml were assayed for lysophospholipase activity. In some of the more active fractions the hydrolysis measured in the fixed time assay fell no longer in the linear range. Therefore, the fractions containing lysophospholipase I were combined and assayed for lysophospholipase activity using several aliquots of the combined fraction to ensure linearity with the amount of enzyme. The total lysophospholipase II activity was determined in the same manner. The proportion of lysophospholipase I and lysophospholipase II was calculated from these measured total activities rather than from peak areas.

From the activities of each of the lysophospholipases and the amounts of protein in the subcellular fractions the relative specific activities for the two lysophospholipases were computed and plotted against the protein distribution (Fig. 3). It is clear from a comparison of Fig. 3 and Fig. 1 that lysophospholipase II parallels closely, the glucose-6-phosphatase distribution. Both enzymes

TABLE II

DISTRIBUTION OF LYSOPHOSPHOLIPASE ACTIVITIES

Activities and specific activities are expressed in m units and m units · mg protein^{-1}, respectively.

Fraction	Total lysophospholipase		Lysophospholipase I		Lysophospholipase II	
	Activity	Spec. act.	Activity	Spec. act.	Activity	Spec. act.
Nuclei, debris	5810	2.71	1300	0.61	4510	2.10
Mitochondria	4280	2.29	1600	0.86	2680	1.43
Lysosomes	2680	6.00	90	0.20	2590	5.80
Microsomes	5310	7,50	80	0.11	5230	7.40
Cytosol	2655	1.06	2360	0.94	292	0.12

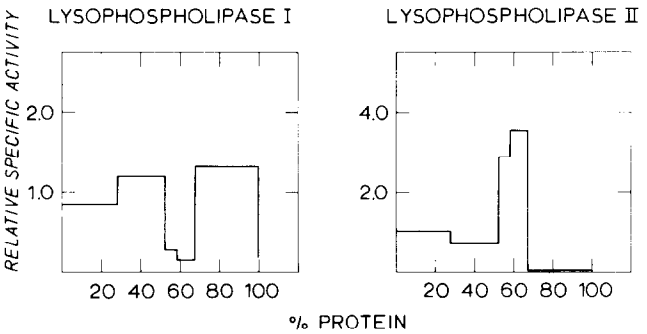

Fig. 3. Calculated relative specific activities of lysophospholipase I and lysophospholipase II versus percentage of total recovered protein in subcellular fractions from beef liver.

have their highest relative specific activity in the microsomal fraction and are virtually absent from the cytosol.

The latter feature distinguishes the lysophospholipase II from that of acid phosphatase. In addition, upon centrifugation of a crude 20 000 × *g* pellet in a sucrose gradient the lysophospholipase II followed the microsomal marker NADPH-cytochrome *c* reductase rather than the lysosomal marker acid phosphatase (Fig. 4). In order to establish whether the lysophospholipase II activity in the 20 000 × *g* pellet and in the mitochondrial fraction can be attributed to microsomal contamination the specific activity of the lysophospholipase II was compared to that of the microsomal markers glucose-6-phosphatase and NADPH-cytochrome *c* reductase (Table III). The specific activity of lysophospholipase II in the mitochondrial and lysosomal fraction amounts to 19 and 78% of that in microsomes. These values are about equal to or even lower than the corresponding figures for the microsomal markers. Therefore, these data strongly suggest that the lysophospholipase II activity in the mitochondrial fraction as well as in the 20 000 × *g* pellet has to be accounted for by microsomal contamination.

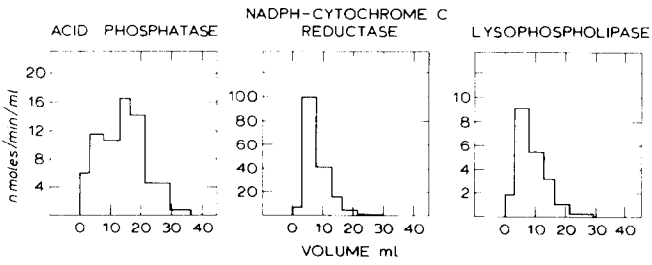

Fig. 4. Distribution of enzymes after sucrose gradient centrifugation of a crude lysosomal fraction. A crude lysosomal fraction (4.0 ml; 52.0 mg protein) in 0.25 M sucrose, 5 mM Tris-HCl (pH 7.2), 2 mM EDTA solution was layered on top of a sucrose gradient and centrifuged as described under Materials and Methods. Seven fractions of the indicated volumes were harvested with the aid of a bent needle connected to a syringe. Left: top fraction of gradient; right: bottom fraction of gradient. Each fraction was analysed for marker enzyme activities and total lysophospholipase activity. The total lysophospholipase activity of the crude lysosomal fraction used in this experiment consisted for 94% of lysophospholipase II. Recoveries from the sucrose gradients were as follows: protein, 95% (distribution not shown); acid phosphatase, 80%; NADPH-cytochrome *c* reductase, 112%; lysophospholipase, 74%.

TABLE III

SPECIFIC ACTIVITIES OF ENZYMES IN SUBCELLULAR FRACTIONS

Specific activities are expressed in nmol · min^{-1} · mg $protein^{-1}$.

Fraction	Lysophospholipase II	Glucose-6-phosphatase	NADPH-cytochrome *c* reductase
Mitochondria	1.43	56.7	6.5
Lysosomes	5.80	153	24.4
Microsomes	7.40	180	33.4

Lysophospholipase I shows a bimodal distribution, with highest relative specific activities in the cytosol and in mitochondria (Fig. 3). This enzyme is found to be virtually absent from the lysosomal and microsomal pellets. Thus, unlike the lysophospholipase II in the crude mitochondrial fraction, which is caused by microsomal contamination, the lysophospholipase I is most likely intrinsic to beef liver mitochondria. If this is correct, further purification of the crude mitochondrial fraction over a sucrose gradient effecting a decrease of the extent of microsomal contamination, should result in a marked increase in the ratio of lysophospholipase I to lysophospholipase II.

In this respect it is worth noting that the crude mitochondrial fraction used for the experiment described in Table IV was prepared just as in the fractionation experiment described in Fig. 1. The endeavor of such fractionation studies is more to determine how a given enzymic activity is quantitatively distributed among subcellular fractions, rather than to obtain these subcellular fractions in the purest state possible. As can be seen in Table IV purification of the crude mitochondrial fraction resulted in a considerable lowering of the microsomal contamination and a concomitant increase in the specific activity of succinate dehydrogenase. The crude mitochondrial fraction contained lysophospholipase I and lysophospholipase II in a proportional ratio of 36 : 64, in good agreement with the results obtained for the crude mitochondrial fraction of the fractionation experiment described in Fig. 2. The purified mitochondria contained essentially only lysophospholipase I (Fig. 5), corroborating the conclusion that the lysophospholipase II activity found in the crude mitochondrial fraction is indeed due to microsomal contamination. Purification of the crude mitochondria resulted in a 37% increase of the specific activity of lysophospho-

TABLE IV

SPECIFIC ACTIVITIES OF LYSOPHOSPHOLIPASE I AND II IN CRUDE AND PURIFIED MITOCHONDRIA

Specific activities are expressed in nmol · min^{-1} · mg^{-1}.

Fraction	NADPH-cytochrome *c* reductase	Succinate dehydrogenase	Total lysophospholipase	Ratio I/II	Lysophospholipase	
					I (calc.)	II (calc.)
Mitochondria, crude	10.8	7.85	3.02	36/64	1.09	1.93
Mitochondria, purified	2.7	9.95	1.59	94/ 6	1.50	0.09
Microsomes	54.0	1.65	7.03	2/98	0.14	6.90

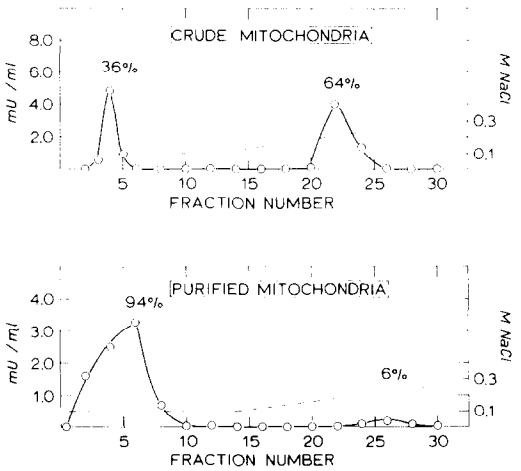

Fig. 5. DEAE-Sephadex chromatography of extracts from crude and purified mitochondria. Crude mitochondria (233 mg protein, 700 m units lysophospholipase activity) were treated with *n*-butanol. After dialysis, the lipid-free extract containing 525 m units lysophospholipase activity was chromatographed over DEAE-Sephadex to yield 146 m units of lysophospholipase I and 260 m units of lysophospholipase II. Likewise, purified mitochondria (248 mg protein, 395 m units) gave a lipid-free extract containing 350 m units lysophospholipase activity. After ion-exchange chromatography 265 m units lysophospholipase I and 18 m units lysophospholipase II were collected.

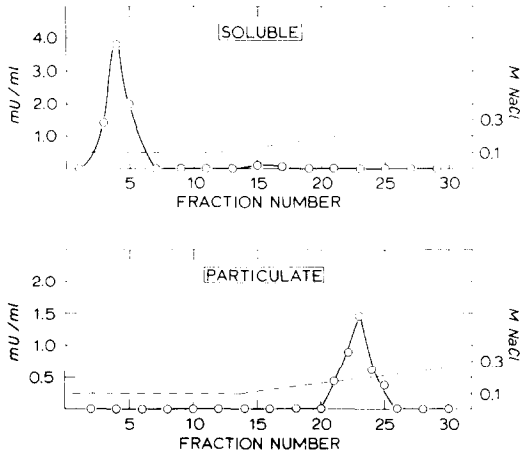

Fig. 6. DEAE-Sephadex chromatography of solubilized proteins from mitochondrial subfractions. Crude mitochondria (149 mg protein, 286 m units lysophospholipase activity) were dialysed overnight against distilled water and separated by centrifugation for 10 min at 20 000 × *g* into a soluble fraction (42 mg protein, 91 m units) and a particulate fraction (103 mg protein, 85 m units). Part of the soluble fraction (82 m units) was chromatographed over the standard DEAE-Sephadex column as described under Materials and Methods to yield 71 m units of lysophospholipase I and no lysophospholipase II (upper figure). Part of the particulate fraction (72 m units) was treated with *n*-butanol to obtain a lipid-free extract. When this extract was chromatographed over a DEAE-Sephadex column 36 m units of lysophospholipase II were obtained. No lysophospholipase I could be detected in the eluate of the ion-exchange column (lower figure).

lipase I in reasonable agreement with the 27% increase in the specific activity of succinate dehydrogenase.

In order to investigate in which submitochondrial fraction the lysophospholipase I is located a crude mitochondrial fraction was dialyzed against destilled water. As can be seen from Fig. 6 the particulate fraction obtained after dialysis contained only lysophospholipase II, which, as earlier experiments have indicated, is caused by microsomal contamination. The absence of the intrinsically mitochondrial lysophospholipase I from the particulate fraction suggests that this enzyme is not a constituent of inner or outer membranes, but instead is part of one of the soluble compartments of mitochondria. In agreement with this hypothesis the soluble fraction obtained after dialysis of mitochondria contained only lysophospholipase I (Fig. 6). Of course, from this experiment it cannot be excluded that the lysophospholipase I is released from mitochondrial inner or outer membranes during the prolonged dialysis against destilled water, although 95% of the recovered succinate dehydrogenase activity was still associated with the particulate fraction. In another experiment a frozen sample of purified mitochondria was thawed and separated immediately in a soluble and a particulate fraction. Even though the mitochondria were kept in isotonic sucrose during this treatment 26% of the lysophospholipase activity, 29% of the malate dehydrogenase activity and 39% of the adenylate kinase activity of the intact mitochondria were released into solution. These results endorse the view that the lysophospholipase I originates from either the matrix inside the mitochondrial inner membrane, like malate dehydrogenase, or from the soluble compartment in between inner and outer membrane, like adenylate kinase.

To further localize lysophospholipase I the release of this enzyme during

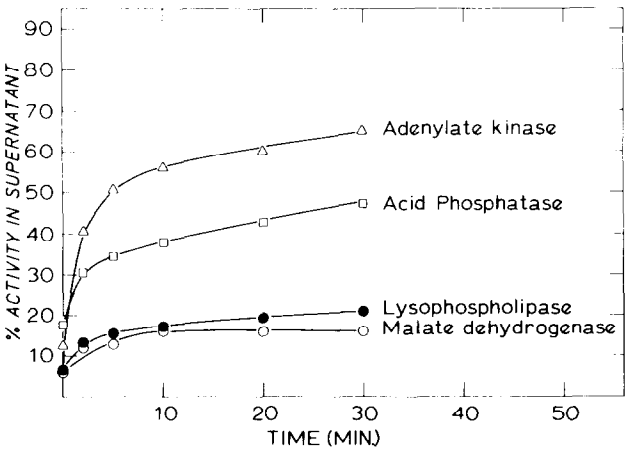

Fig. 7. Treatment of purified mitochondria in hypotonic medium. Mitochondria (15.8 mg protein) in 1.0 ml of 250 mM sucrose containing 5 mM Tris-HCl buffer (pH 7.2) and 2 mM EDTA were diluted with 4.0 ml of 20 mM Tris-HCl buffer (pH 7.2) containing 1 mM EDTA. The mixtures were incubated at 37°C for the indicated time periods and separated into a soluble and a particulate fraction by centrifugation for 10 min at 20 000 × *g*. The particulate fraction was resuspended in 20 mM Tris-HCl buffer (pH 7.2) containing 1 mM EDTA. Aliquots of the soluble and particulate fractions were assayed for the indicated enzymatic activities. The percentage leak on vertical axis indicates the amount of enzymic activity found in the soluble fraction expressed as percent of total recovered activity in soluble and particulate fractions. Total recoveries were always better than 90%.

hypotonic treatment of intact purified mitochondria, essentially as described by Pfaff and Schwalbach [25], was compared with the release of the above mentioned marker enzymes for each of the soluble compartments. Although the results described in Fig. 3 argued strongly already against a lysosomal localization of lysophospholipase I the release of acid phosphatase from lysosomes still contaminating the purified mitochondria was also measured in this experiment. The results (Fig. 7) further endorse the view that lysophospholipase I is not located in lysosomes, but instead is present in the same compartment that accommodates the mitochondrial malate dehydrogenase, i.e. the mitochondrial matrix.

A mitochondrial localization of lysophospholipase I is also in line with the results obtained after centrifugation of a crude mitochondrial fraction over a sucrose gradient. As can be seen from Fig. 8 the lysophospholipase I was found to be distributed over the gradient fractions as was observed for the mitochondrial marker succinate dehydrogenase and unlike the lysosomal marker acid phosphatase. It is also important to note from this experiment that lysophospholipase II followed much more closely the distribution of the microsomal NADPH-cytochrome *c* reductase than the lysosomal acid phosphatase. This confirmed the data presented in Fig. 4.

The bimodal distribution of lysophospholipase I as observed in Fig. 3, raised the question whether perhaps the presence of this enzyme in the cytosol fraction was an artefact due to rupture of the mitochondria during homogenization of the beef liver. Although such an explanation is very unlikely in view of the higher relative specific activity of lysophospholipase I in the cytosol

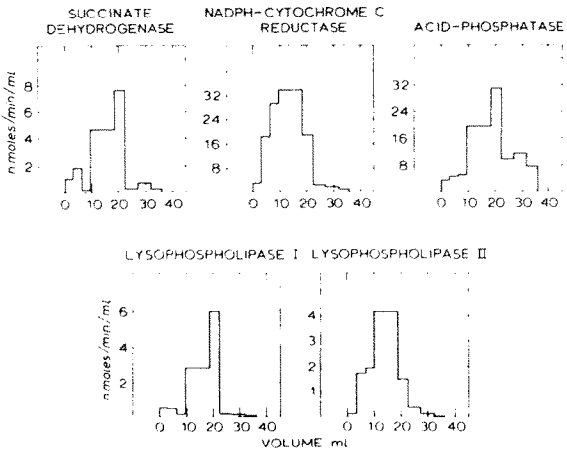

Fig. 8. Distribution of enzymes after sucrose gradient centrifugation of a crude mitochondrial fraction. A crude mitochondrial fraction (4.0 ml; 109.6 mg protein) was layered on top of a sucrose gradient and centrifuged as described under Materials and Methods. Eight fractions of the indicated volumes were harvested as described in the legend of fig. 4 and analyzed for marker enzyme activities and total lysophospholipase activity. The contribution of lysophospholipase I and II to the total lysophospholipase activity in each fraction was determined after solubilization of the enzymes and separation over mini DEAE-Sephadex columns in Pasteur pipets. Recoveries from the sucrose gradient were as follows: protein, 90% (distribution not shown); succinate dehydrogenase, 72%; NADPH-cytochrome *c* reductase, 78.5%; acid phosphatase, 79%; total lysophospholipase, 113%; lysophospholipase I, 71%; lysophospholipase II, 81%.

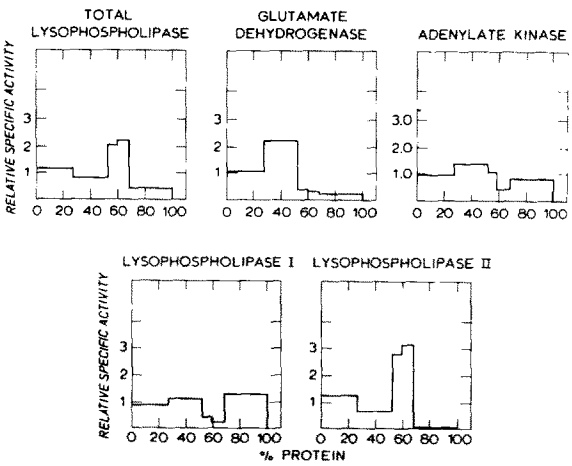

Fig. 9. Relative specific activities of lysophospholipase, adenylate kinase and glutamate dehydrogenase versus percentage of total recovered protein in subcellular fractions from beef liver. Compare legend of Fig. 1. Total recoveries after cell fractionation were as follows: protein, 95%; glutamate dehydrogenase, 75%; adenylate kinase, 94%; total lysophospholipase, 99%; lysophospholipase I, 115%; lysophospholipase II, 91%.

fraction, the possibility was checked by comparing the distribution of lysophospholipase I with that of glutamate dehydrogenase. The latter enzyme was shown to be a useful marker for rat liver mitochondria by Beaufay et al. [16], in that this enzyme does not occur in an extramitochondrial cytoplasmic form, although the presence of a different glutamate dehydrogenase in nuclear membranes, at least for ox liver, has been reported [26]. Mitochondrial glutamate dehydrogenase is exclusively present in the matrix fraction [17] and the appearance of this enzyme in the cytosolic fraction can thus serve as an indication for rupture of mitochondria. A new fractionation experiment was therefore carried out in which the distribution of lysophospholipase I over the various subcellular fractions was compared with that of glutamate dehydrogenase. Note the good agreement between the distribution of lysophospholipases in Fig. 9 and the data presented for these enzymes in Figs 1 and 3. The appearance of lysophospholipase I in the cytosolic fraction cannot be explained by a leak of this enzyme from ruptured mitochondria. Although the distribution of adenylate kinase indicated a considerable damage of the mitochondrial outer membrane, glutamate dehydrogenase appeared to a much lower degree in the 100 000 × *g* supernatant. Thus, by combining these results with those of Fig. 7, lysophospholipase I is believed to occur in both the mitochondrial matrix and the cytoplasm.

Discussion

In a previous communication [10] beef liver was shown to contain two enzymes with lysophospholipase activity. Both proteins were purified to homogeneity and partly characterized. Lysophospholipase I had an estimated molecular weight of 25 000 and an isoelectric point of 5.2, whereas the correspond-

ing data for lysophospholipase II amounted to 60 000 and 4.5, respectively. In this paper the localization of both enzymes, as determined by classical cell fractionation studies, is reported. Of course, the interpretation of such fractionation studies depends on the assumption that the lysophospholipase from a given subcellular compartment distributes in a similar way as the marker enzyme(s) for that compartment, thus not taking into account any possible specific absorption phenomena. Within these limitations, lysophospholipase I appeared to be a soluble enzyme with a bimodal distribution, i.e. occurring in the mitochondrial matrix and in the cytoplasma. Such a distribution has been reported for various enzymes, e.g. glycerol-3-phosphate dehydrogenase [27], β-hydroxybutyrate dehydrogenase [28], NADPH-linked isocitrate dehydrogenase [28], L-malate dehydrogenase [29,30], aldehyde dehydrogenase [31,32] and glutamate-aspartate-amino-transferase [30]. Usually, however, the mitochondrial and cytoplasmic form can be distinguished quite easily as distinct biochemical and catalytic species by either physical techniques and/or a differenct co-factor requirement. Within the limitations of the fractionation experiments described in this paper the possibility that both lysophospholipase I activities originate from the same subcellular compartment has been excluded. The relative specific activity of lysophospholipase I in the mitochondrial fraction is much too high, in comparison to that of glucose-6-phosphate dehydrogenase (Figs 3 and 1), to be accounted for by contamination with the cytosol. Conversely, the relative specific activity of lysophospholipase I in the cytosol fraction is much too high, in comparison with that of glutamate dehydrogenase (Fig. 9), to explain the occurrence of lysophospholipase I in the cytosol by leakage from the mitochondria. At present it is unknown whether lysophospholipase I from mitochondria is completely identical to the enzyme from the cytoplasma. Although we have never observed a separation of lysophospholipase I into two peaks during purification of this enzyme from whole beef liver, this problem is now under investigation by purifying the enzyme from both the mitochondrial pellet and the cytoplasmic fraction.

Lysophospholipase II appeared to be a membrane bound enzyme with highest relative specific activity in the microsomal fraction. The different subcellular localization of lysophospholipase I and lysophospholipase II corroborates the conclusion from the previous paper [10] that lysophospholipase I and lysophospholipase II are completely different enzymes. Sodium dodecyl sulfate disc electrophoresis under strongly reducing conditions indicated that no simple monomer to dimer- or trimer relationship exists between these two enzymes. Also preliminary immunological experiments (unpublished observations) indicate that lysophospholipase I and II are distinct protein species.

The possibility that the cytoplasmic lysophospholipase I (M_r, 25 000) is a soluble part of the membrane-bound lysophospholipase II (M_r, 60 000), artificially formed by the action of endogenous proteolytic enzymes during the homogenization and centrifugation procedures, was considered. A beef liver homogenate was therefore kept for various periods at room temperature (up to 22 h) and then analyzed for lysophospholipase activities. Standing at room temperature resulted in a gradual decrease of total lysophospholipase activity (28% decrease after 22 h), but the ratio of lysophospholipase I to II remained essentially constant at 0.25 and no increase of lysophospholipase I activity at

the expense of lysophospholipase II activity was observed.

Whether the two different lysophospholipases have different physiological functions remains obscure at present. It is tempting to consider the membrane-bound lysophospholipase II as being involved in the degradative part of the turnover of membrane phosphoglycerides. Experiments are designed to study this and to see whether also the soluble lysophospholipase I is active against membrane-bound lysophosphoglycerides. Concerning the function of the mitochondrial lysophospholipase I the observation of Honjo and Ozawa [33] that lysolecithin inhibits electron transport in the region between flavoprotein and coenzyme Q may be relevant.

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