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STUDIES ON LYSOPHOSPHOLIPASES

III. THE COMPLETE PURIFICATION OF TWO PROTEINS WITH LYSOPHOSPHOLIPASE ACTIVITY FROM BEEF LIVER

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Summary

1. Two proteins with lysophospholipase activity (EC 3.1.1.5), provisionally denoted lysophospholipase I and lysophospholipase II, were found to be present in beef liver homogenates. These lysophospholipases were well separated during DEAE—cellulose chromatography of delipidated beef liver extracts.

2. Lysophospholipase I and lysophospholipase II required a 3590-fold and a 770-fold purification, respectively, in order to obtain homogeneous preparations.

3. The molecular weights of lysophospholipases I and II were estimated to be about 25 000 and 60 000, respectively. Isoelectric points of 5.2 and 4.5 were measured for the two enzymes.

4. In accordance with previous observations on beef pancreatic lysophospholipase, both lysophospholipases from beef liver exhibited general esterolytic properties in that compounds like tributyrin and *p*-nitrophenylacetate were also hydrolysed. Evidence is provided indicating that the hydrolysis of these compounds is an intrinsic property of the beef liver lysophospholipases.

5. Highly purified non-specific carboxylesterases (EC 3.1.1.1) were found to be essentially inactive against 1-acyllysophosphatidylcholine.

Introduction

Lysophospholipases (EC 3.1.1.5) are thought to play an essential role in the catabolism of phosphoglycerides by preventing accumulation of the lytic intermediary lysophosphoglycerides. Despite the widespread occurrence of lysophospholipases (for a review see [1]) the enzyme was only recently obtained in a homogeneous form from beef pancreas [2]. Moreover, a homo-

geneous protein, initially purified as a phospholipase A_1 from *Escherichia coli*, was found to be more active on lysophosphoglycerides than on phosphoglycerides [3]. Studies on the substrate specificity of the pancreatic enzyme revealed it to exhibit not only phospholipase A_1 activity [4], but also general esterolytic properties in that compounds containing carboxylester bonds such as short chain triglycerides and *p*-nitrophenylacetate were also hydrolysed [5]. The pancreatic enzyme is excreted, however, and it is quite conceivable that the broad substrate specificity of this enzyme is related to other physiological functions than participation in the catabolism of membrane phosphoglycerides. We have, therefore, isolated the lysophospholipase activity of beef liver. After we had solubilized the enzymic activity by *n*-butanol treatment of a beef liver homogenate and chromatographed the soluble protein fraction on a DEAE—Sephadex column two protein peaks with lysophospholipase activity emerged well separated from the column. The enzymes eluting at about 0.05 M and 0.2 M NaCl were provisionally denoted lysophospholipase I and lysophospholipase II, respectively. In this paper we report on the complete purification of lysophospholipase I (3600-fold) and lysophospholipase II (770-fold). In addition some properties of both enzymes are described.

Materials and Methods

Protein techniques. Fresh beef liver was obtained from the local slaughterhouse. All manipulations during protein purification were carried out at 0—4°C. Polyacrylamide disc gel electrophoresis was performed according to Ornstein and Davies [6] on 3.5% gels. For sodium dodecylsulfate disc electrophoresis the method described by Shapiro et al. [7] was used employing 5% gels. Isoelectric focusing was carried out in a LKB 8101 electrofocusing column with LKB ampholines as described by Vesterberg [8]. Protein was determined according to Lowry et al. [9]. If protein were to be determined in the presence of β-mercaptoethanol the latter compound was first carboxylmethylated with iodoacetate according to Ross and Schatz [10].

Enzyme assays. Lysophospholipase activity was assayed as described previously [2]. The assay mixtures contained 0.4 mM 1-[1-^{14}C] acyl-glycero-3-phosphoryl choline in 20 mM potassium phosphate buffer pH 7.5 for lysophospholipase I and pH 7.0 for lysophospholipase II. The assay mixture for lysophospholipase I contained in addition 2 mM β-mercaptoethanol.

Enzymic activity versus tributyrin was measured by titration of the liberated fatty acids with a Radiometer pH-stat TTT_2 equipment as described previously [2]. *p*-Nitrophenylacetate hydrolysis was measured spectrophotometrically with the aid of an Unicam SP 500 at a substrate concentration of 0.33 mM [2].

Substrates. 1-[1-^{14}C] Acyl-glycero-3-phosphorylcholine was a mixture of synthetic 1-[1-^{14}C] palmitoyl-glycero-3-phosphorylcholine and hydrogenated 1-acyl-glycero-3-phosphorylcholine prepared from egg lecithin. The final product had a specific activity of 50 000 dpm ^{14}C per μmole phosphorus. Phosphorus was determined by the method of Chen et al. [11] on samples ashed according to Ames and Dubin [12].

1-[1-^{14}C]Palmitoyl-glycero-3-phosphorylcholine was prepared by acylation of the $CdCl_2$-adduct of glycerylphosphorylcholine with [1-^{14}C] palmitoylchloride and subsequent degradation of the purified di-[1-^{14}C] palmitoylphosphatidylcholine with *Crotalus adamanteus* phospholipase A_2 as described previously [13].

Tributyrin was a product of Fluka AG, Buchs, Switzerland. *p*-Nitrophenylacetate was obtained from Merck AG, Darmstadt, G.F.R.

Results

Purification of lysophospholipase I

The results of a typical purification are summarized in Table I. Step 1. Fresh beef liver (1 kg) was homogenized in 2 l of 20 mM Tris—HCl (pH 7.3) containing 0.15 M NaCl during 2 min in a Waring blendor. The homogenate was filtered through two layers of cheese cloth. Step 2. An equal volume of cold *n*-butanol saturated with water was added to the homogenate. The mixture was stirred vigorously for two min with a glass rod and then centrifuged immediately for 20 min at 10 000 rev./min in the GSA rotor of a Sorvall RC-2B centrifuge. The upper butanol layer was removed with a Pasteur pipet connected to an aspirator. The water layer was filtered through cheese cloth to yield a clear butanol extract. This extract was dialyzed against three batches of 4 vols each of 20 mM Tris—HCl buffer pH 7.3. During dialysis an inactive precipitate was formed which was removed by centrifugation. Step 3. The clear supernatant was applied to a DEAE—cellulose column (Whatman DE 23; bed volume 700 ml, 7 cm diameter) equilibrated with the dialysis buffer. The lysophospholipase I was somewhat retarded on this column but could be eluted with this buffer. Lysophospholipase II was only eluted after application of a salt gradient (compare the result of a pilot experiment on DEAE—Sephadex in Fig. 1). The ratio of lysophospholipase I to lysophospholipase II varied from 0.17—0.24 in 5 experiments. Assuming that the recoveries for both enzymes during steps 2 and 3 were the same, the specific activities, total yield and purification factors for both enzymes could be calculated in the homogenate and the butanol extract. Step 4. The combined fractions containing lysophos-

TABLE I

PURIFICATION OF LYSOPHOSPHOLIPASE I FROM BEEF LIVER

Purification step		Protein (mg)	Activity (units)	Specific activity (mU/mg)	Recovery (%)	Purification (-fold)
1.	Cell-free homogenate	184000	72*	0.39	100	—
2.	*n*-Butanol extract	37900	47*	1.24	65	3.2
3.	DEAE—cellulose DE 23	6300	40	6.3	55	16.1
4.	40—60% $(NH_4)_2SO_4$	3400	39	11.5	54	29.5
5.	Sephadex G-100	483	31	64	43	164
6.	DEAE—cellulose DE 52	39	23.9	610	33	1564
7.	CM—cellulose CM 52	25	17.9	720	25	1846
8.	Sephadex G-50	8	11.2	1400	15	3590

* Calculated, see text.

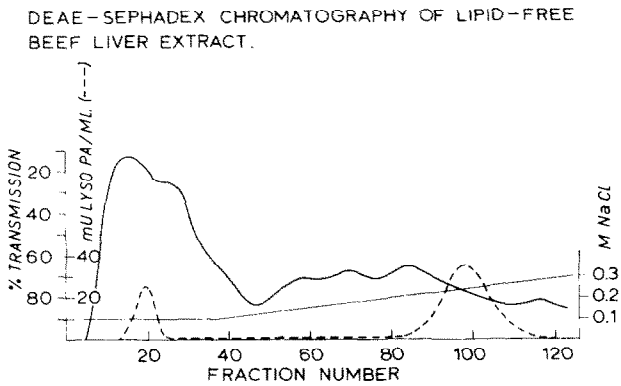

Fig. 1. Chromatography of lipid-free beef liver extract on DEAE—Sephadex A-50. A lipid-free extract (2700 mg protein) obtained as described in step 2 of the purification procedures was made 0.1 M with respect to NaCl and applied to a DEAE—Sephadex A-50 column (dimensions, 10 · 4.5 cm) equilibrated with 20 mM Tris—HCl buffer, pH 7.3, containing 0.1 M NaCl. The column was washed with 600 ml of this buffer and then eluted with a gradient of 650 ml each of 0.1 M and 0.3 M NaCl in the Tris—HCl buffer. Fractions of 18 ml were collected at a flow rate of 54 ml/h.

pholipase I were brought to 60% saturation with $(NH_4)_2SO_4$ and stirred for 45 min. The precipitate was collected by centrifugation and dissolved in 50 ml 20 mM Tris—HCl buffer (pH 7.3) containing 0.15 M NaCl. Step 5. The solution obtained in step 4 was applied to a Sephadex G-100 column (dimensions 150 · 3 cm) and eluted with the same buffer. The active fractions were dialysed against 20 mM Tris—HCl buffer (pH 7.3) containing 10 mM β-mercaptoethanol. Step 6. The dialysate was chromatographed on a second DEAE—cellulose column (Whatman DE 52 microgranular, dimensions 60 · 2.5 cm). The lysophospholipase I was again eluted from this column with the equilibrating buffer. Step 7. Attempts to purify the lysophospholipase I by cation exchange column chromatography were unsuccessful due to great losses of activity under conditions where the enzyme is bound to the column. The enzyme was therefore further purified in a CM—cellulose batch experiment. The fraction from the previous step was dialysed against 30 vols of 80 mM sodium acetate buffer (pH 5.0) containing 10 mM β-mercaptoethanol. Ten g of CM—cellulose (Whatman CM 52 microgranular) were added to the dialysate. The mixture was stirred for 30 min and then centrifuged to remove the CM—cellulose. Step 8. As lyophilization caused great losses of enzymic activity the supernatant of step 7 was concentrated by ultrafiltration (Amicon diaflo, UM 2 membrane) to one seventh of the original volume. This solution was then brought to 90% saturation with $(NH_4)_2SO_4$. The precipitate obtained after centrifugation was dissolved in 2.5 ml 20 mM Tris—HCl buffer (pH 7.3) containing 0.15 M NaCl and 10 mM β-mercaptoethanol. This solution was filtered through a Sephadex G-50 column (dimensions 100 · 1.5 cm) equilibrated with the same buffer. The fractions with constant specific activity were collected to yield a nearly homogeneous preparation representing a 3590-fold purified enzyme. This preparation lost activity by repeated freezing and thawing but could be stored at −20° C in 10 mM Tris—HCl buffer (pH 7.3), containing 5 mM β-mercaptoethanol and 50% glycerol for several months without appreciable loss of activity.

TABLE II

PURIFICATION OF LYSOPHOSPHOLIPASE II FROM BEEF LIVER

Purification step		Protein (mg)	Activity (units)	Specific activity (mU/mg)	Recovery (%)	Purification (-fold)
1.	Cell-free homogenate	166000	294*	1.77	100	—
2.	*n*-Butanol extract	23300	144*	6.2	49	3.5
3.	DEAE—Sephadex	816	135	165	46	93
4.	Hydroxylapatite	197	117	595	40	336
5.	Sephadex G-100	55	72.6	1320	25	745
6.	Sephadex G-100	48	65.3	1360	22	770

* Calculated, see text.

Purification of lysophospholipase II

The purification procedure for lysophospholipase II starting from 1000 g of beef liver is summarized in Table II. Steps 1 and 2 are identical to those described for the purification of lysophospholipase I. Step 3. The butanol extract was dialyzed against 20 mM Tris—HCl buffer (pH 7.3) containing 0.1 M NaCl and applied to a DEAE—Sephadex A-50 column (bed volume 280 ml) equilibrated with the same buffer. The column was rinsed with this buffer till the eluate contained virtually no protein. A linear gradient of 5 bed volumes of each 0.1 M and 0.3 M NaCl in 20 mM Tris—HCl buffer (pH 7.3) was then applied to the column. The lysophospholipase II was eluted at about 0.18—0.23 M NaCl. The fractions containing this enzymic activity were combined and dialyzed against 10 mM potassium phosphate buffer (pH 7.0). Step 4. The dialysate from the previous step was pumped onto a hydroxylapatite column (Biogel HTP, Biorad Labs., Richmond, Calif., bed volume 260 ml) equilibrated with 10 mM potassium phosphate buffer (pH 7.0). After the column was washed with 160 ml of this buffer, a linear gradient of 5 bed vols each of 10 mM and 100 mM potassium phosphate buffer (pH 7.0) was

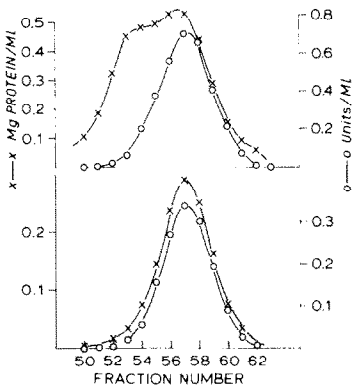

Fig. 2. Sephadex G-100 filtration of lysophospholipase II. Upper figure: 50 mg of the protein fraction obtained from the hydroxylapatite column (step 4, Table II) was filtered through a Sephadex G-100 column (15 · 3 cm) as described in step 5 of the purification procedure for lysophospholipase II. Lower figure: 15 mg protein obtained from fractions 56—61 of the first Sephadex G-100 column were applied to the same column. Fractions of 8 ml were collected at a flow rate of 25 ml/h.

applied. At about 55 mM—85 mM potassium phosphate one protein peak coinciding with lysophospholipase II activity was eluted. The combined fractions were dialyzed against distilled water and lyophilized. Step 5. The lyophilized powder was dissolved in a small volume of 20 mM Tris—HCl buffer (pH 7.3) containing 0.15 M NaCl and percolated through a Sephadex G-100 column (150 · 3 cm). The elution pattern is indicated in the upper part of Fig. 2. The second of two, only partially resolved, protein peaks contained the lysophospholipase activity. Fractions 53—55 and 56—61 were combined and both fractions were then dialyzed against distilled water and lyophilized. The purity of both fractions was determined by polyacrylamide disc gel electrophoresis (Fig. 3). The hydroxylapatite fraction obtained in step 4 consisted of two

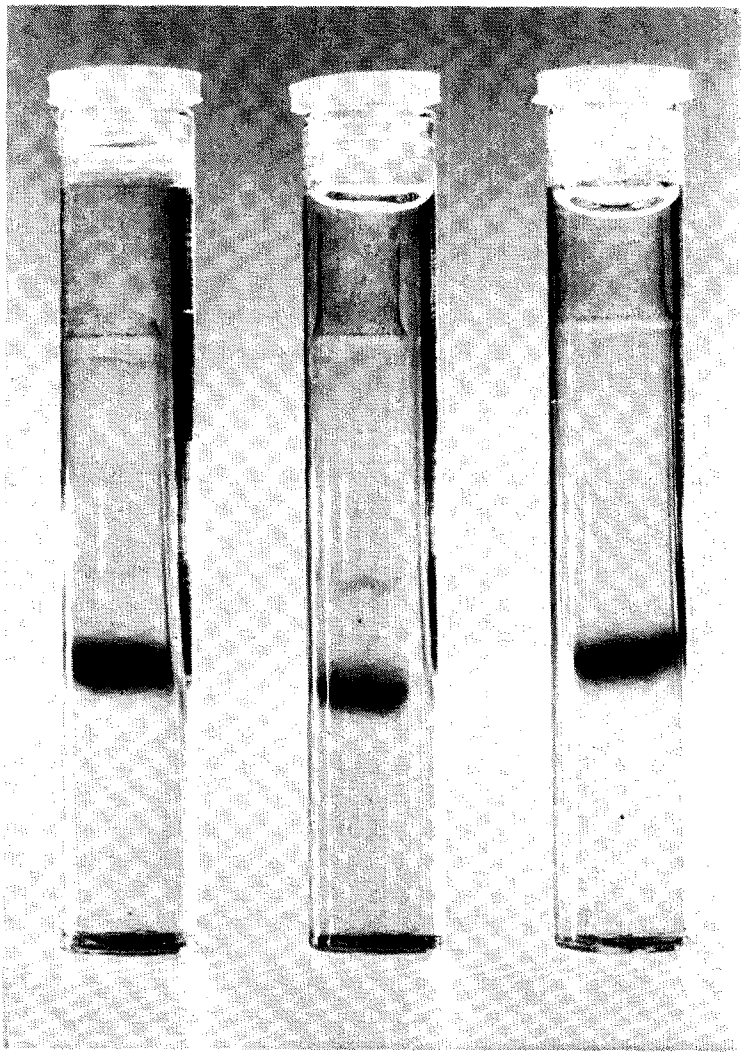

Fig. 3. Polyacrylamide disc electrophoresis of lysophospholipase II. Electrophoresis was carried out on 3.5% gels employing a constant current of 4 mA per tube at 4°C. Electrophoresis buffer: 50 mM Tris 0.38 M glycine, pH 8.3. Cathode at top. Left: 20 μg of hydroxylapatite fraction. Middle: 20 μg of the combined fractions 53—55 from the first Sephadex G-100 column. Right: 10 μg of the combined fractions 56—61 from the first Sephadex G-100 column (compare Fig. 2). Protein bands were stained in a solution of 1% Amido black in 7% acetic acid.

protein bands. The lower one corresponded to the protein eluting in fractions 53—55 of the Sephadex G-100 column and the upper band corresponded to the lysophospholipase II eluting in fractions 56—61 of the Sephadex column. Obviously, the lysophospholipase II was still contaminated with the protein eluting slightly earlier from the Sephadex G-100 column. Many attempts to separate the two proteins in the hydroxylapatite fraction by ion exchange chromatography or isoelectric focusing were unsuccessful as could be expected from their very similar behaviour on disc electrophoresis. Step 6. The lyophilized powder from fractions 56—61 of the first Sephadex column was used for a second filtration through the same column under the conditions described in step 5. This resulted in the elution of an almost symmetrical protein peak coinciding with lysophospholipase activity (Fig. 2, lower part.). The fractions with constant specific activity were combined and used for experiments to characterize lysophospholipase II.

Purity

Polyacrylamide-gel electrophoresis under non-denaturing conditions on 3.5% gels gave one band for lysophospholipase I and lysophospholipase II. In agreement with the behaviour of both enzymes on DEAE-exchange columns the lysophospholipase II showed a much higher migration rate (Fig. 4, left part). Also, sodium dodecylsulfate disc gel electrophoresis on samples reduced in the presence of β-mercaptoethanol or dithiothreitol gave one band for both enzymes. In this system the lysophospholipase I migrated further than lysophospholipase II (Fig. 4, right part).

Molecular weight

The molecular weight of lysophospholipases I and II were estimated to be about 24 000 and 63 000 respectively from sodium dodecylsulphate-disc gel electrophoresis (Fig. 5). Filtration of both enzymes through a Sephadex G-100 column previously calibrated with proteins of known molecular weights yielded estimated molecular weights of 26 000 and 57 000 for lysophospholipase I and lysophospholipase II, respectively (data not shown).

Substrate specificity

In view of the general esterolytic properties of pancreatic lysophospholipase [3], we have investigated the activity of the purified beef liver lysophospholipases versus tributyrin and *p*-nitrophenylacetate. Both lysophospholipase preparations from beef liver exhibit esterolytic properties comparable to the pancreatic enzyme, although important variations can be noted in the relative rates of hydrolysis of the investigated substrates for the different enzymes (Table III). Nevertheless, the results of these limited specificity studies provoke the question whether lysophospholipase activity is simply a side-action of non-specific carboxylesterases (EC 3.1.1.1). In this respect it is interesting to note that as yet no physiological function has been assigned to these non-specific carboxylesterases [14]. In order to answer the question whether lysophospholipase activity (EC 3.1.1.5) is caused by non-specific carboxylesterases, several highly purified carboxylesterases were tested for lysophospholipase activity. The results of these assays (Table IV) clearly show the high specific

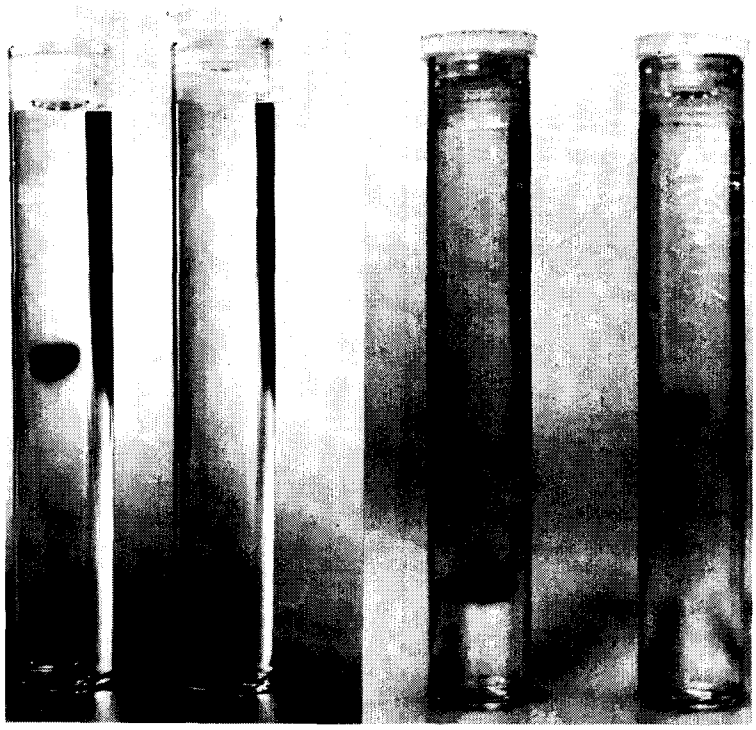

Fig. 4. Polyacrylamide disc electrophoresis of purified lysophospholipases I and II. From left to right: 10 μg of lysophospholipase I and 10 μg of lysophospholipase II (both normal discs); 30 μg of lysophospholipase I and 20 μg of lysophospholipase II (both sodium dodecylsulfate discs). Electrophoresis on normal discs was carried out at 4°C during 45 min as described in the legend of Fig. 3. For the sodium dodecylsulfate disc electrophoresis the proteins were reduced and denatured in 1% sodium dodecylsulfate and 1% β-mercaptoethanol in 0.1 M potassium phosphate buffer (pH 7.1) during 2 min at 100°C. Electrophoresis was carried out for 4 h at a constant current of 5 mA per tube with the cathode at the top. The gels were fixed in 12.5% trichloracetic acid for 1 h at 50°C, washed several times with methanol—acetic acid—water (5 : 1 : 5, v/v) and then stained with 0.25% Coomassie Brilliant Blue R-250 in this mixture.

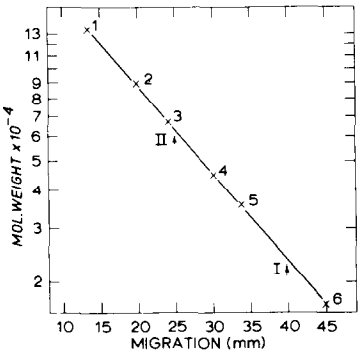

Fig. 5. Estimation of molecular weights of lysophospholipases by sodium dodecylsulfate disc electrophoresis. The procedure described in the legend of Fig. 4 was used except that the electrophoresis of 20 μg of each of the proteins was carried out for 3 h and 15 min. (1) Bovine serum albumin, dimer; (2) Ovalbumin, dimer; (3) Bovine serum albumin (Armour Pharmaceutical Co. Ltd, Eastbourne, England); (4) Ovalbumin (Worthington Biochem. Corp., Freehold, N.J. U.S.A.); (5) Pepsin (Serva, Heidelberg, G.F.R.); (6) Myoglobin (Mann Res. Labs., U.S.A.); I, Lysophospholipase I; II, Lysophospholipase II.,

TABLE III

ESTEROLYTIC PROPERTIES OF LYSOLECITHIN HYDROLYZING ENZYMES

Enzyme	Specific activity (I.U./mg)		
	1-Acyl lysolecithin	Tributyrin	*p*-Nitrophenyl-acetate
Beef pancrease lysophospholipase	6.2	49	20
Beef liver lysophospholipase I	1.40	3.8	2.1
Beef liver lysophospholipase II	1.36	268	11

activities of these carboxylesterases towards *p*-nitrophenylacetate and tributyrin. None of the esterases tested, however, showed any appreciable activity in hydrolysing lysolecithin. Thus, it can be concluded that lysophospholipase activity is not simply due to a side-action of non-specific carboxylesterases, although the lysophospholipase preparations isolated sofar certainly exhibit general esterolytic properties. It has been a major concern to show that these esterolytic properties are intrinsic to the lysophospholipases from beef liver and that the hydrolysis of *p*-nitrophenyl-acetate and tributyrin by the lysophospholipase preparations is not caused by a small contamination with a very active carboxylesterase. Isoelectric focusing, one of the most potent techniques in protein purification, was used to investigate this problem. When a crude butanol extract from beef liver was subjected to isoelectric focusing in an ampholine gradient from pH 4—6 the two lysophospholipase peaks were well separated from the main very active carboxylesterase peak (Fig. 6; note scale differences). An isoelectric point of pH 5.2 was found for the lysophospholipase I and of pH 4.5 for the lysophospholipase II. The lysophospholipase II activity coincided with a small peak of esterase activity versus *p*-nitrophenyl-acetate, which apparently is not due to tailing off of the main carboxylesterase activity as intermediate fractions contained zero esterase activity. The esterase activity of lysophospholipase I in this experiment was too low to be measured accurately. This is in agreement with the lower ratio of esterase to lysophospholipase activity which is 1.5 for lysophospholipase I in contrast to a value of 8 for lysophospholipase II (compare Table IV). When the pure lysophospholipase I was isoelectrofocused on the same column the esterase activity of this

TABLE IV

LACK OF LYSOPHOSPHOLIPASE ACTIVITY OF CARBOXYLESTERASES

Enzyme source	Specific activity (I.U./mg)		
	1-Acyl lysolecithin	Tributyrin	*p*-Nitrophenyl-acetate
Pig liver*	0.00	79	63
Pig liver **	0.07	286	211
Beef liver **	0.00	24	182

* Commercial preparation (Boehringer, Mannheim, GFR)

** Kindly donated by Professor Dr K. Krisch, Kiel, GFR.

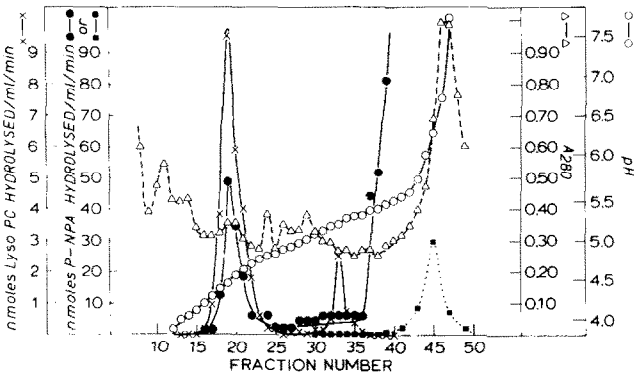

Fig. 6. Isoelectric focusing of lipid-free beef liver extract. Isoelectric focusing was carried out during 68 h at 500 V in an LKB 8101 column using ampholytes (LKB Produkter AB, Sweden) for the pH range of 4—6 with the cathode at the top. The column was loaded with 18 mg protein from a lipid-free beef liver extract prepared as described in step 2 of the purification procedure. Fractions of 2 ml were collected and assayed for lysophosphatidylcholine (lyso-PC) and *p*-nitrophenylacetate (P-NPA) hydrolysing activity. Hydrolysis of *p*-nitrophenylacetate is expressed in nmoles · ml^{-1} · min^{-1} (●——●) or in nmoles · ml^{-1} · min^{-1}. 10^{-2} (■——■) with the cathode at the right.

preparation against *p*-nitrophenylacetate was recovered in the fractions containing lysophospholipase I activity (Fig. 7) and not in the less acidic region were the main peak of beef liver carboxylesterase activity banded (compare Fig. 6).

Isoelectric focusing of purified lysophospholipase II again showed the esterase activity to coincide with lysophospholipase II activity (Fig. 8). The amounts of protein used for the isoelectric focusing experiments described in Fig. 7 and 8 were too low to allow accurate determination of the protein contents of the fractions in the presence of ampholytes and sucrose. However, the high purity of lysophospholipase I and II as judged from the different disc gels and the results of the isoelectric focusing experiments clearly indicate that the hydrolysis of *p*-nitrophenylacetate is an intrinsic property of these lysophospholipases. In this respect it is worth mentioning that also during the final Sephadex filtration experiments in the purification of these enzymes lyso-

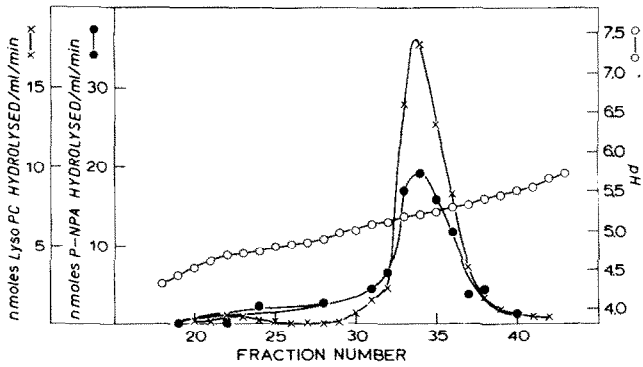

Fig. 7. Isoelectric focusing of purified lysophospholipase I. Conditions as for Fig. 6. The column was loaded with 0.16 mg purified lysophospholipase I.

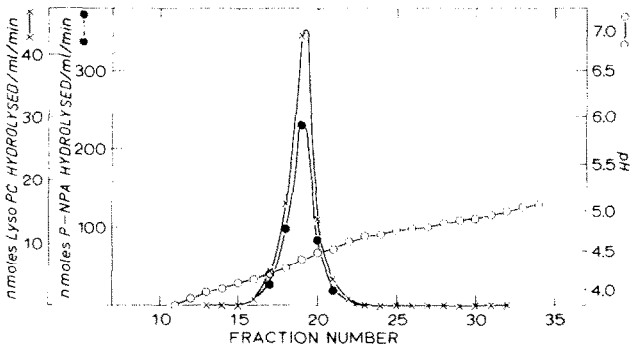

Fig. 8. Isoelectric focusing of purified lysophospholipase II. The isoelectric focusing was carried out on 0.20 mg of purified lysophospholipase II. Same conditions as in Fig. 6.

phospholipase activity coincided with *p*-nitrophenylacetate hydrolysing activity (data not shown).

Properties of lysophospholipase I and II

The hydrolysis of 1-acyllysophosphatidylcholine by lysophospholipase II was easily shown to be linear with time and protein concentrations under the standard assay conditions used. More difficulties were encountered in obtaining such linearities with lysophospholipase I. This turned out to be due to a loss of activity of this enzyme during the incubation. Also in preliminary experiments low recoveries were obtained during the last purification steps. Both problems could be solved by handling the enzyme in the presence of β-mercaptoethanol. Assays were routinely done in the presence of 2 mM β-mercaptoethanol, whereas the purification of this lysophospholipase I from step 5 on was carried out in buffer solutions containing 10 mM β-mercaptoethanol. The loss of activity of the enzyme during incubation could also be overcome by addition of 1 mM EDTA. This reagent was used to replace β-mercaptoethanol in experiments in which the influence of SH-reagents on the activity of lysophospholipase I was investigated (see Table VI).

The deacylation of 1-acyllysophosphatidylcholine by lysophospholipase I showed a broad pH optimum between pH 6 and 8. The pH-activity curve for lysophospholipase II was sharper, with an optimum at pH 8.5.

Neither lysophospholipase required Ca^{2+} or other bivalent cations as is evident from the almost negligible inhibition by EDTA (Table V). In accord with previous observations on impure [15–21] as well as on highly purified lysophospholipases [2] both beef liver lysophospholipases appear to be several inhibited by detergents such as sodium deoxycholate and Triton X-100, although the lysophospholipase I is somewhat less sensitive to such detergents. Bis-[*p*-nitrophenyl]phosphate, a known inhibitor of carboxylesterases [14] inhibited lysophospholipase II to a much greater extent than it did lysophospholipase I. In addition, lysophospholipase II activity is completely abolished by the presence of 1 mM diisopropylfluorophosphate. Lysophospholipase I is much less sensitive to this agent, although it is not yet possible to state that this enzyme is completely resistant to diisopropylfluorophosphate.

TABLE V

EFFECT OF VARIOUS AGENTS ON LYSOPHOSPHOLIPASE ACTIVITY

Addition	Concentration	% Inhibition	
		Lysophospholipase I	Lysophospholipase II
Ca^{2+}	1 mM	0	0
EDTA	1 mM	2	9
sodium deoxycholate	1 mg/ml	70	99
Triton X-100	0.5 mg/ml	33	90
bis-[*p*-nitrophenyl]phosphate	0.01mM	8	61
	0.04mM	11	95

Both lysophospholipases are inhibited by SH-reagents although in general lysophospholipase I appears to be more sensitive to SH-reagents than lysophospholipase II (Table VI). In this respect the beef liver enzymes differ from the pancreatic enzyme [2] which was not inhibited at all at the concentrations of the SH-reagents indicated in Table VI. The reactivity of SH-groups in the lysophospholipases and their importance for enzymic activity will be investigated in more detail in future experiments.

Discussion

In order to establish the pathway for the catabolism of phosphoglycerides in cells or subcellular fractions often radioactively labelled phospholipids were incubated with the enzyme source under investigation. From an analysis of the degradation products it has become clear that the sequential deacylation of phosphoglycerides by a phospholipase A and a lysophospholipase is one of the major pathways to account for the catabolic part of phosphoglyceride turnover [22]. The involvement of a lysophospholipase activity in this process was sometimes shown by using labelled lysophospholipids, but not infrequently the presence of a lysophospholipase in the preparation was inferred from a less than stoichiometric accumulation of lysophosphoglycerides. Using these criteria lysophospholipases have been described to occur throughout nature (for a review see [1]) but the substrate specificity of this type of lipolytic enzyme has not been investigated adequately due to a lack of purified enzymes.

The findings reported in this paper with regard to the substrate specificity

TABLE VI

EFFECT OF SH-REAGENTS ON LYSOPHOSPHOLIPASE ACTIVITY

Addition	Concentration (μM)	% Inhibition	
		Lysophospholipase I	Lysophospholipase II
Dithionitrobenzoic acid	400	47	15
N-ethylmaleimide	10000	88	15
Iodoacetamide	20000	47	15
p-Hydroxymercuribenzoate	10000	93	67

of the two lysophospholipase activities from beef liver corroborate the earlier results obtained on purified beef pancreatic lysophospholipase [5]. All these enzymes are not specific for lysophosphoglycerides, but in addition catalyze the hydrolysis of carboxylester bonds in various other substrates. These observations raised the question of whether the hydrolysis of lysophospholipids is caused by non-specific carboxylesterases. This question can be answered negatively as several purified non-specific carboxylesterases exhibited no appreciable lysophospholipase carboxylesterase (Table IV). In addition the carboxylesterase activity of the lysophospholipases, when measured towards a typical carboxylesterase substrate such as *p*-nitrophenylacetate, amounted to only a few percent of that observed with non-specific carboxylesterases (compare Tables III and IV). Thus, either the lysophospholipases are still contaminated with small amounts of highly active carboxylesterases or the hydrolysis of *p*-nitrophenylacetate is an intrinsic property of lysophospholipases.

The first possibility was ruled out by the results of isoelectric focusing experiments (Figs. 6, 7 and 8). These experiments clearly indicated that the main carboxylesterase peak in beef liver extract was well separated from lysophospholipases I and II and that the esterase activities in the fractions containing the lysophospholipases could not be explained on the basis of tailing of the main esterase peak. The position of this peak with an isoelectric point greater than pH 5.4 agree well with the results of Wynne and Shalitin [23] who reported an isoelectric point of pH 5.5 for beef liver esterase. These authors found only one peak with esterase activity in beef liver. On the other hand Benöhr and Krisch [24] observed a second protein with esterase activity after high voltage electrophoresis on starch gels. This protein had a somewhat lower isoelectric point than the main beef liver carboxylesterase. It is interesting to note that the esterase activity associated with the lysophospholipases also has a lower isoelectric point than the main beef liver carboxylesterase.

When the purified lysophospholipase I and II were electrofocused individually, the esterase activity coincided in each case with the lysophospholipase activity. In these experiments (Fig. 7 and 8) no esterase activity was found in the fractions where the main beef liver carboxylesterase was expected to peak (compare Fig. 6), indicating that the esterolytic activity of the purified lysophospholipases is not due to contamination with beef liver carboxylesterase.

These results indicate that hydrolysis of *p*-nitrophenylacetate is an intrinsic property of the beef liver lysophospholipases. Hydrolysis of the ester bond in *p*-nitrophenylacetate appears to be an intrinsic property of many proteins, either hydrolases such as chymotrypsin [25], and lysozyme [26] or other enzymes such as 3-phosphoglyceraldehyde dehydrogenase [27], glycero-3-phosphate dehydrogenase [28], aldehydedehydrogenase [29] and carbonic anhydrase [30]. It is known from the work of Piszkiewicz and Bruice [26] that the splitting of *p*-nitrophenylacetate by hen's egg white lysozyme takes place at an esteratic site which differs from the catalytic site for the hydrolysis of the main substrate. Likewise, the esterolytic and dehydrogenase activities of glycero-3-phosphatedehydrogenase probably do not share the same active site [28]. Experiments with active site directed agents such as diisopropylfluorophosphate and bis-[*p*-nitrophenyl]phosphate will be needed to establish whether lysophospholipid deacylation and *p*-nitrophenylacetate hydrolysis are catalysed by the same active center in the isolated lysophospholipases.

The protein with lysophospholipase activity isolated from beef pancreas [2] was shown to exhibit phospholipase A_1 activity under certain assay conditions [4]. The maximal phospholipase A_1 activity versus long chain phosphatidylethanolamine amounted to about 8% of the optimal lysophospholipase activity versus 1-palmitoyl lysolecithin. Both lysophospholipases from beef liver, when tested on doubly-labelled 1-[9,10-$^3H_2$]palmitoyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine under various conditions, always showed phospholipase A_1 activities which were less than 2% of the respective lysophospholipase activities. Phospholipase A_2 activity could not be detected in these preparations. A study of the action of the purified lysophospholipases on a homologous series of phosphatidylcholines and lysophosphatidylcholines with different saturated acyl chains is in progress.

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