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Authors
Zhang, YY
Dennis, EA

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Purification and Characterization of a Lysophospholipase from a Macrophage-like Cell Line P388D$_1$*

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Yingyi Zhang and Edward A. Dennis‡

From the Department of Chemistry, University of California, San Diego, La Jolla, California 92093

Two lysophospholipase activities (designated I and II) were identified in the macrophage-like cell line P388D$_1$. Lysophospholipase I was purified (8,500-fold) to homogeneity by DEAE-Sephacel, Sephadex G-75, Blue-Sepharose, and chromatofocusing chromatography. Lysophospholipase II was separated from the lysophospholipase I in the Blue-Sepharose step. The apparent molecular mass of lysophospholipase I and II are 27,000 and 28,000 daltons, respectively, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Their pI values were 4.4 and 6.1, respectively, as determined by isoelectric focusing. Lysophospholipase I exhibited a broad pH optimum between 7.5–9.0. The double-reciprocal plot of the substrate dependence curve of the purified lysophospholipase I showed a break around the critical micelle concentration of the substrate (1-palmitoyl-*sn*-glycerol-3-phosphorylcholine). The apparent K_m, determined from substrate concentrations above 10 μM was 22 μM, and the apparent V_{max} was 1.3 μmol min^{-1}mg^{-1}. The purified enzyme did not have phospholipase A$_1$, phospholipase A$_2$, acyltransferase, or lysophospholipase-transacylase activity. No activity was detected toward triacylglycerol, diacylglycerol, *p*-nitrophenol acetate, *p*-nitrophenol palmitate, or cholesterol ester. The enzyme did, however, hydrolyze monoacylglycerol although at a rate 20-fold less than lysophospholipid, 0.06 μmol min^{-1}mg^{-1}. The lysophospholipase I was inhibited by fatty acids but not by glycerol-3-phosphorylcholine, glycerol-3-phosphorylethanolamine, or glyc-fjerol-3-phosphorylserine. A synthetic manoalide analogue 3(*cis,cis*,-7,10)hexadecadienyl-4-hydroxy-2-butenolide inhibited the enzyme with half-inhibition (IC$_{50}$) at about 160 μM. Triton X-100 decreased the enzymatic activity, although this apparent inhibition can be explained by a "surface dilution" effect. The pure lysophospholipase I was stable for at least 5 months at −20 °C in the presence of glycerol and β-mercaptoethanol. Lysophospholipid also demonstrated a protective effect during the later stage of purification.

Lysophospholipids are detergent-like intermediates of phospholipid metabolism that exhibit potent cytolytic and membrane-perturbing effects (1). Accumulation of lysophospholipids *in vivo* is therefore strictly regulated, and at least five different types of enzymes are directly involved in producing or removing them as shown in Fig. 1. Included among these are lysophospholipases and phospholipase As. Our laboratory has identified and separated several phospholipases from the macrophage-like cell line P388D$_1$ (2). Among these a membrane-bound Ca^{2+}-dependent phospholipase A$_2$ was further purified and characterized (3). However, during the early stages of that study, it was found that lysophospholipase activity in the broken cells was much higher than the phospholipase A$_2$ activity (2). Similar ratios of activities had previously been observed in human amniotic tissue (4) and rabbit myocardium (5).

Lysophospholipases are likely to be important enzymes in a number of physiological and pathological processes. Aside from direct lytic effects, their substrates, lysophospholipids, have been associated with a wide range of phenomena, such as induction of lethal dysrhythmias in myocardial infarction (6), segmental demyelination of peripheral nerves (7), and stimulation or inhibition of several important enzymes (8–12). Most relevant to our studies is the fact that lysophospholipids are thought to stimulate macrophage phagocytosis (13–15). A key question, however, is what is the relative contribution of the lysophospholipase *versus* the other enzymes involved in lysophospholipid turnover. The macrophage-like cell line P388D$_1$ provides an opportune system in which to study these competing reactions in an intact cell. We, therefore, undertook the purification and characterization of its lysophospholipases preliminary to our long term goal of studying these enzymes in the intact cell.

The work reported herein represents the first characterization of a lysophospholipase from a mammalian cell line. Only a few lysophospholipases have been purified from mammalian tissues (4, 5, 16, 17) and among these, two have wider substrate specificities and also serve as general esterases (17, 18). As shown here, two lysophospholipase activities can be distinguished in the macrophage-like cell line P388D$_1$. We have now been able to purify one completely stable form of lysophospholipase and found it to be highly specific for lysophospholipids.

EXPERIMENTAL PROCEDURES

Materials—1-[1-^{14}C]Palmitoyl-*sn*-glycero-3-phosphorylcholine and 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine were purchased from Amersham Corp. ^{14}C-Labeled glycerides were the gift of Dr. John Khoo (University of California, San Diego, School of Medicine). 1-Palmitoyl-*sn*-glycerol-3-phosphorylcholine was purchased from Avanti Polar Lipids (Birmingham, AL). L-α-Lysophosphatidylcholine, type I (used for column buffer), palmitic acid, stearic acid, oleic acid, arachidonic acid, L-α-glycerophosphorylcholine (Grade V), L-α-glycerophosphorylethanolamine, L-α-glycerolphosphorylserine, phenylmethylsulfonyl fluoride, bis-(*p*-nitrophenyl) phosphate, and polyethylene glycol compound (M_r 15,000–20,000) were purchased from Sigma. DEAE-Sephacel, Sephadex G-75, Sephadex G-75 superfine, Blue-Sepharose CL-6B, Poly Buffer Exchange 94 and Polybuffer 74 were obtained from Pharmacia LKB Biotech-

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‡ To whom correspondence should be addressed.

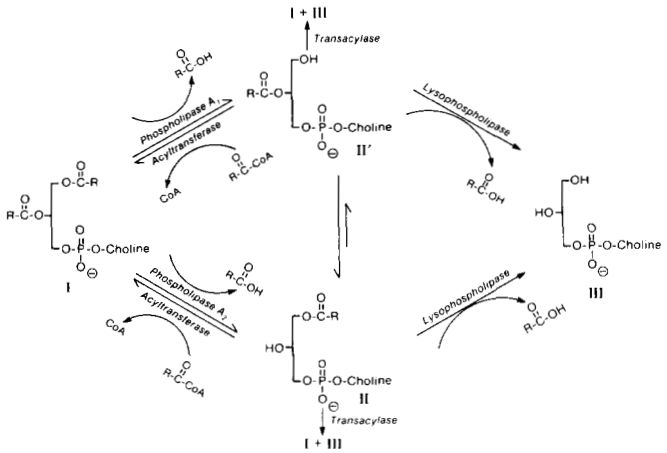

FIG. 1. **Possible metabolic interconversions of lysophosphatidylcholine, phosphatidylcholine (*I*), 1-acyl-lysophosphatidylcholine (*II*), 2-acyl-lysophosphatidylcholine (*II'*), and glycerolphosphorylcholine (*III*).** The interconversions of II and II' via a nonenzymatic acyl migration is also shown. The transacylase combines two molecules of lysophosphatidycholine to form one molecule of phosphatidylcholine and one molecule of glycerolphosphorylcholine.

nology Inc. Bio-Rad protein assay kit I were purchased from Bio-Rad. Spectra/Por 2 dialysis tubing was purchased from Spectrum Medical Industries, Inc. (Los Angeles, CA). Silica-Gel G (250 μM) plates were purchased from Analtech, Inc. (Newark, DE). HDHB[1] was provided by Dr. Edward Mihelich, Lilly Research Laboratories (19). All other reagents were analytical grade or better.

Purification Procedures—The P388D$_1$ cells were grown, harvested, and broken by nitrogen cavitation as described by Ross *et al.* (2). The homogenate was centrifuged at 1000 × *g* at 4 °C for 15 min. The supernatant (LS-1) was collected and stored at −20 °C for use in the lysophospholipase purification described herein. Generally, 1×10^{10} cells (from 20 roller bottles of cell culture) yielded about 280 ml of LS-1. Except for the DEAE-Sephacel column, the same size columns have been used for preparations resulting from 1×10^{10} to 2×10^{10} cells.

All of the following steps were carried out at 4–8 °C. LS-1 was thawed and adjusted to pH 8.3 with 1 N NaOH and loaded onto a DEAE-Sephacel column previously equilibrated with 10 mM Tris-HCl, pH 8.3, 2 mM EDTA, and 10 mM β-mercaptoethanol (BME). The column was eluted with equilibration buffer containing a 0–0.24 M NaCl gradient. Optimum column resolution was obtained with a column bed volume to sample volume ratio of 1:1.6. A slow flow rate (6-cm h^{-1}) was required to efficiently remove large amounts of turbid material. The active fractions from the void volume and the beginning of the gradient were pooled together.

To concentrate the protein a single 80% saturation ammonium sulfate cut was taken by adding solid ammonium sulfate to the pooled DEAE fractions over a 20-min period. The mixture was stirred slowly for another 30–40 min, and the precipitate was pelleted by centrifugation at 12,000 × *g* for 30 min. The pellet was resuspended in 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 10 mM BME and immediately loaded onto a Sephadex G-75 column (2.5 × 100 cm) previously equilibrated with 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 5 mM BME. The protein was eluted with equilibration buffer at a flow rate of 20 ml h^{-1}.

The active fractions pooled from the Sephadex G-75 column were adjusted to pH 6.0 with 2% acetic acid and immediately loaded onto a Blue-Sepharose column (1.5 × 20 cm) previously equilibrated with 10 mM Tris-HAc and 1 mM EDTA, pH 6.0. The column was then washed with one volume of the equilibration buffer and eluted with a pH gradient from pH 6.0 to 8.5 with 25 mM imidazole-HCl buffer containing 10 mM BME, 10% glycerol, and 10 μM lyso-PC. The pooled fractions from the first active peak (Blue I) were adjusted with 0.1 M NaOH to pH 7.5 and loaded onto a chromatofocusing column.

The chromatofocusing column (1 × 20 cm) was packed with Polybuffer Exchange 94 and equilibrated with 25 mM imidazole-HCl buffer, pH 7.5, containing 10 mM BME, 10% glycerol, and 10 μM lyso-PC. After the sample loading the column was washed with one volume of the equilibration buffer and eluted with a 1:8 dilution of Polybuffer 74 at pH 4.5 containing 10 mM BME, 10% glycerol, and 10 μM lyso-PC. The fractions (5 ml) were collected into 1.5 ml of 0.15 M imidazole buffer, pH 7.5, containing 10 mM BME, and 8 mM EDTA. The active fractions were pooled and placed in Spectra/Por 2 dialysis tubing (12,000–14,000 molecular weight cutoff) concentrated against 70–80% (w/v) polyethylene glycol compound/H_2O. The concentrate was then loaded onto a Sephadex G-75 superfine column (2.5 × 50 cm) equilibrated with 25 mM imidazole-HCl buffer, pH 7.5, containing 30 mM NaCl, 10% glycerol, 10 mM BME, 2 mM EDTA, and 10 μM lyso-PC. The active fractions from this column were immediately placed into Spectra/Por 2 dialysis tubing and immersed in 100% glycerol containing 10 mM BME at 4 °C for 2–3 h. This purified enzyme solution was stored at −20 °C.

Assay of Lysophospholipase Activity—The assay mixture contained 0.5 ml of 0.1 M Tris-HCl, pH 8.0, 125 μM [^{14}C]lyso-PC (0.2 μCi/μmol) and 5–10 μl (40–80 ng) of purified enzyme or a comparable amount of activity of a partially purified preparation. The incubation was carried out at 40 °C for 30 min. Enzymatic activity was determined by one of two methods. One was based on the modified Dole extraction (20). The other employed a modified Bligh and Dyer extraction (21) followed by separation of the reactants and products by TLC.

In the Dole assay the reaction was stopped by the addition of 2.5 ml of 2-propanol/heptane/1 N H_2SO_4 (20:5:1, v/v/v). About 0.1 mg of silica gel (Bio-sil A, 100–200 mesh, Bio-Rad) was added and vortexed immediately. Then 1.5 ml of heptane and 1.5 ml of deionized water were added and vortexed for at least 10 s. One ml of the upper phase was mixed with 5 ml of scintillation fluid and counted.

In the TLC assay the reaction was stopped by the addition of 0.5 ml of chloroform/methanol/glacial acetic acid (2:4:1, v/v/v), followed by the addition of 0.25 ml of chloroform. The lower phase was removed and dried under vacuum at 40 °C. The residue was dissolved in 30 μl of chloroform/methanol/glacial acetic acid (2:4:1, v/v/v). The entire sample was spotted onto silica gel G plates, and the reaction products were separated by elution with chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v). The lipids were visualized with I_2 vapor, and the zones corresponding to fatty acid and lyso-PC were scraped directly into scintillation vials and counted with 6 ml of scintillation fluid.

The efficiency of the extraction used in the above assays was determined by extracting and counting solutions that contained known amounts of fatty acid and lysophospholipid. The TLC assay overestimated the percentage of fatty acid present by about 2–4% while the Dole assay underestimated it by about 5–9%. There may also be a very small decrease in extraction efficiency at higher lyso-PC and fatty acid concentration. Both of these effects were caused by incomplete extraction of lysophospholipid or fatty acid. The average deviation of duplicate or triplicate experimental points was always less than ±5% in both assays. However, overall, the TLC assay is more accurate and reliable than the Dole assay, although the former assay is much more laborious than the latter one. All kinetic studies were carried out with the TLC assay while the Dole assay was used to monitor the purification.

Phospholipase A_1 and A_2 Assay—The phospholipase A_1 and A_2 activities were assayed according to the procedure described by Ulevitch *et al.* (3). The assay mixture contained 5 mM $CaCl_2$, 20 mM glycine buffer, pH 9.0, and 100 μM dipalmitoyl PC containing about 100,000 cpm of 1-palmitoyl-2-[1-^{14}C]palmitoyl PC. Dipalmitoyl PC was prepared as vesicles by sonicating the phospholipid in buffer and Ca^{2+} using an MSE model 100-watt sonicator until the lipid suspension clarified (about 5 min). The reaction was started by the addition of 10 μl of the enzyme solution (80 ng of protein) to give a final volume of 0.5 ml. The assays were then incubated at 40 °C for 30 min. The lipid products were extracted and separated on TLC. The zones corresponding to fatty acid, dipalmitoyl PC, and lyso-PC were scraped and counted.

Acyltransferase Assay—For the assay of acyltransferase activity, the assay mixture contained 125 μM 1-[1-^{14}C]palmitoyl lyso-PC (0.2 μCi/μmol), 125 μM palmitoyl coenzyme A, or oleoyl coenzyme A and 2 mM $MgCl_2$. The reaction was started by the addition of 10 μl of purified enzyme (80 ng) to give a final volume of 0.5 ml. The assays were then incubated at 40 °C for 30 min. The lipid products were extracted and separated as in the lysophospholipase assay. The for-

[1] The abbreviations used are: HDHB, 3(*cis,cis*-7,10)hexadecadienyl-4-hydroxy-2-butenolide; CMC, critical micelle concentration; BME, β-mercaptoethanol; lyso PC, palmitoyl lysophosphatidylcholine; PC, phosphatidylcholine; TLC, thin layer chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

mation of PC was attributed to acyltransferase activity.

Transacylase Assay—The transacylase activity was assayed using the same assay mixture as employed in the lysophospholipase assay. The lipid products were extracted and separated on TLC. Since the transacylase uses two molecules of lyso-PC to form one molecule of PC and one molecule of glycero-3-phosphorylcholine, the enzyme activity was determined by formation of [^{14}C]PC.

Lipase and Cholesterol Esterase Assay—Mono-, di-, triglyceride lipase, and cholesterol esterase activities were determined according to the method of Khoo and Steinberg (22). The substrate in each case was mono-, di-, trioleoylglycerol or oleoylcholesterol labeled with [1-^{14}C]oleic acid. The substrates were dissolved in benzene, dried under N_2, and redissolved in ethanol. The substrate/ethanol solution (2% ethanol) was then mixed with water while vortexing. The assay mixture contained 100 μM substrate (1.6 μCi/μmol), 0.1 M Tris-HCl, pH 8.0, 0.03% BSA, and enzyme in a total volume of 0.8 ml. The mixture was incubated at 40 °C for 30 min and then stopped by vortexing with 3 ml of methanol/benzene/chloroform (2.4:2:1, v/v/v) containing 0.1 mM oleic acid. The mixture was then vortexed with 0.1 ml of 1 M NaOH and then centrifuged at 2000 × *g* for 5–10 min. A 1.0-ml aliquot of the aqueous layer was removed for counting.

Esterase Assay—Esterase activity was tested toward *p*-nitrophenol acetate and *p*-nitrophenol palmitate according to the method of Huggins and Lapides (23). The substrates were first dissolved in methanol and then diluted in aqueous solution. The assay mixture contained 68 μM of substrate, 10 mM of Tris-HCl, pH 8.0, and 80 ng of purified lysophospholipase I. The mixture was incubated at 22 °C for 1 h. The product release was detected by the measurement of the increase in absorbance at 400 nm. Since BME and EDTA alone can catalyze the hydrolysis of these substrates, the elution buffer of the last Sephadex G-75 column was included in the control sample. Cholesterolesterase was used as positive control.

Protein Determination—During the earlier purification steps, the protein concentration was determined by the Bradford dye-binding procedure (24) using the Bio-Rad protein assay kit I. Because the column buffers interferes with this assay, the protein concentration of Blue I and II and the active pool from the chromatofocusing column were determined from a protein standard curve made by dilution of the protein standard with the respective column elution buffer. The concentration of the final purified enzyme was measured by the TCA precipitation-Lowry protein assay of Bensadoun and Weinstein (25). Bovine γ-globulin was used as the protein standard in both methods.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (26). A 5.2% stacking gel and a 12% separating gel with 37.5/1 (A/B) were used. Aliquots from the active pool in each purification step were heated at 100 °C for 5 min with 25% volume of 0.5 M Tris-HCl buffer containing 20% bovine serum albumin, 40% glycerol, and 4% SDS. The slab gel was run at 15 mA. Proteins were visualized by silver staining (27).

Isoelectric Focusing—Isoelectrofocusing was performed in a glycerol gradient. The dense solution contained 54% glycerol and 4% ampholyte. The less dense solution contained 20% enzyme solution and 0.1% BME. The basic solution was 0.25 M NaOH. The acid solution contained 63% glycerol and 0.15 M H_3PO_4. The isoelectric focusing column was run at 1 watt and 1200 V for 3 days. The focused material was removed from the column with a peristaltic pump at a flow rate of 0.4 ml min^{-1}. The pH and activity were then measured on each of the collected fractions.

RESULTS

Enzyme Purification—The result of the purification of a typical preparation of cells is summarized in Table I. The column chromatographic steps are illustrated in Fig. 2. Two to three active peaks were recovered on the DEAE-Sephacel column (Fig. 2*A*). The first and second active peaks eluted in the void volume, and the third active peak appeared at the very beginning of the NaCl gradient. The appearance of the first peak was not always reproducible. In some preparations only one active peak appeared in the void volume. None of the three active peaks contained lysophospholipase-transacylase activity. It was found to be most efficient to pool the active peaks together at this stage and separate them again (see "Discussion") on the Blue-Sepharose column. With N_2 cavitation of the P388D$_1$ cells, large quantities of nucleic acids are present in LS-1. Precipitation of the nucleic acids with streptomycin was not successful because some of the lysophospholipase coprecipitated with the nucleic acids during centrifugation, but the DEAE-Sephacel column removed the nucleic acid efficiently. The active fractions from the DEAE-Sephacel column were precipitated with ammonium sulfate and loaded onto a Sephadex G-75 column (Fig. 2*B*). While the purification with this column was only 3-fold, it served to remove the ammonium sulfate and lower the BME concentration as BME prevented the enzyme from binding to the Blue-Sepharose. This procedure was employed rather than dialysis because the latter resulted in a 30% loss of enzymatic activity.

Two forms of lysophospholipases, designated lysophospholipase I and lysophospholipase II were completely separated on the Blue Sepharose column (Fig. 2*C*). The lysophospholipase I (Blue I peak) was eluted with the buffer change at pH = 6.0 and the lysophospholipase II (Blue II peak) was eluted at about pH = 7.0. The combined total yield of lysophospholipase I and II was 67%. The Blue I was loaded onto a chromatofocusing column and eluted as a single peak of activity at pH 5.5 (Fig. 2*D*). The A_{280} profile is not shown in Fig. 2*D* since the protein concentration was very low, and the column equilibration buffer had more absorption at A_{280} than the protein. Since the enzyme was unstable at pH values lower than 6, the fractions were collected directly into a pH 7.5 buffer. The pH profile was determined in a separate experiment. The active pool from the chromatofocusing column was concentrated and loaded onto a Sephadex G-75 superfine column from which a single active peak (lysophospholipase I) eluted at about 30,000 daltons.

The SDS-PAGE gel of the protein at various stages of purification is shown in Fig. 3. The final yield of the purification procedure was 8.6% with a 8,500-fold purification. Various preparations have yielded specific activities between 1.3 and 1.7 μmol min^{-1}mg^{-1}. The further purification of Blue II on similar columns was not as successful because the

TABLE I

Purification of lysophospholipase from the macrophage-like cell line P388D$_1$

Step	Total protein	Total activity	Yield	Specific activity	Purification
	mg	*nmol min^{-1}*	*%*	*nmol min^{-1} mg^{-1}*	*fold*
1. Homogenate	15,300	2,410	100	0.158	1
2. LS-1	6,530	1,190	49	0.182	1.2
3. DEAE-Sephacel	808	955	40	1.18	7.5
4. Sephadex G-75	193	737	31	3.82	24
5. Blue-Sepharose (I)	3.50	341	14	97.4	620
Blue-Sepharose (II)	5.40	149	6.2	27.6	180
6. Chromatofocusing of I	0.321	288	12	897	5,700
7. Sephadex G-75	0.154	206	8.6	1,340	8,500

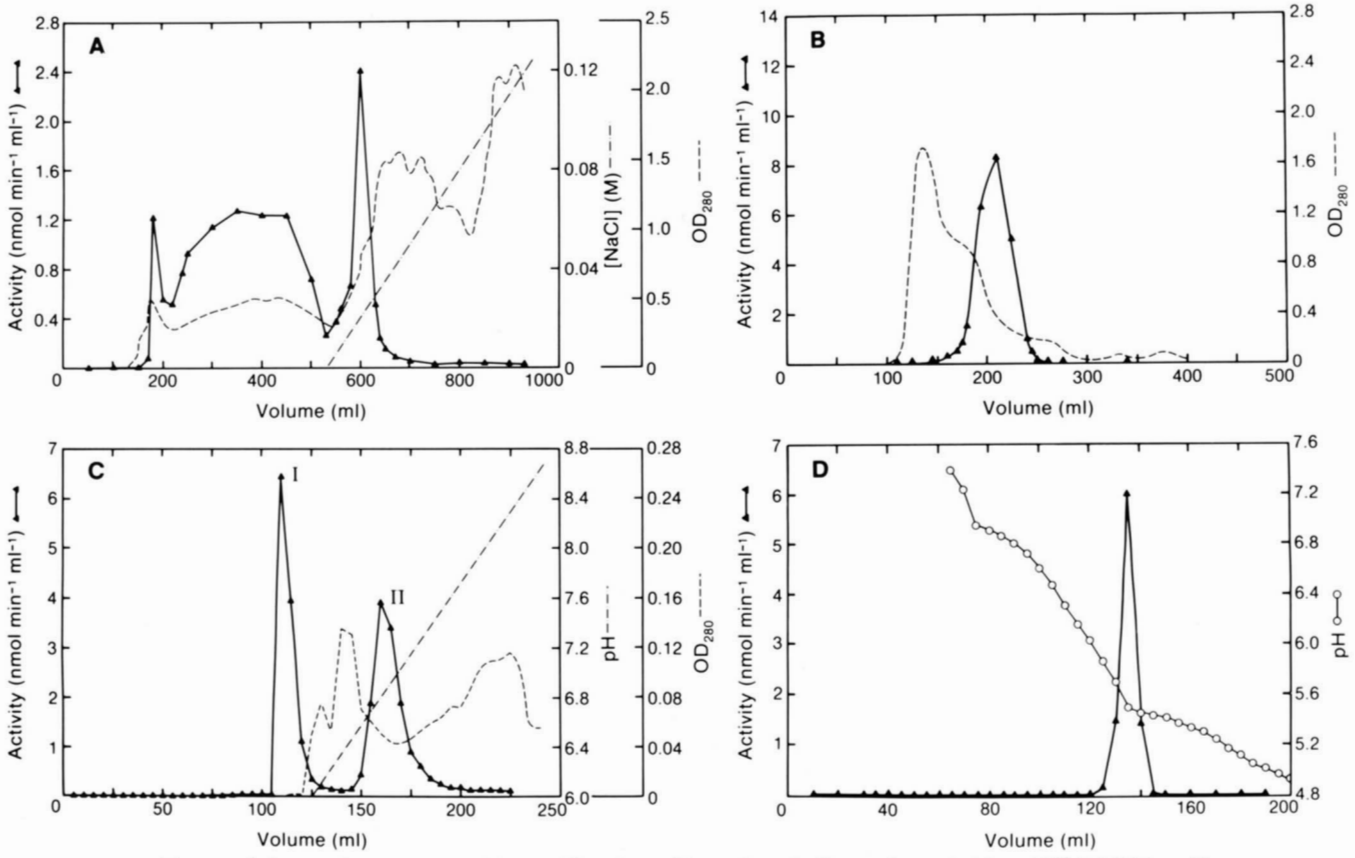

FIG. 2. **Column chromatographic purification of lysophospholipase from 1.16 × 10^{10} P388D$_1$ cells.** *A*, DEAE-Sephacel column (2.5 × 40 cm). This column was loaded with 325 ml of LS-1 and eluted with a 0–0.12 M NaCl gradient. *B*, Sephadex G-75 column (2.5 × 90 cm). *C*, Blue-Sepharose column (1.5 × 20 cm). The first active peak, I, is referred to as Blue I and the second active peak, II, is referred to as Blue II. *D*, chromatofocusing column (1 × 10 cm). The optical density of the protein was not detectable. The pH measurement of the eluate was carried out in a separate experiment.

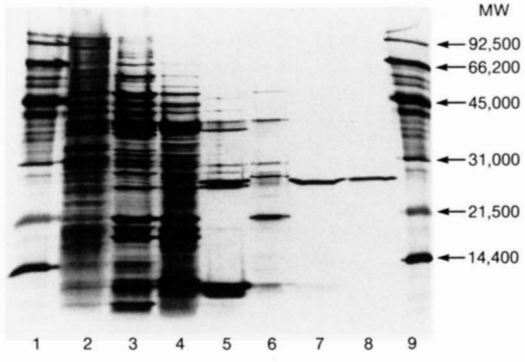

FIG. 3. **SDS-polyacrylamide gel electrophoresis of the P388D$_1$ lysophospholipase.** *Lanes 1* and *9* are molecular weight standards; *lane 2*, 26 μg of LS-1 protein; *lane 3*, 31 μg of the pooled active fractions from the DEAE-Sephacel column; *lane 4*, 34 μg of the pooled active fractions from the Sephadex G-75 column; *lane 5*, 8 μg of the Blue I peak from the Blue Sepharose column; *lane 6*, 2.5 μg of the Blue II peak from the Blue-Sepharose column; *lane 7*, 1.5 μg of the pooled active fractions from the chromatofocusing column; *lane 8*, 0.8 μg of the pooled active fractions from the final Sephadex G-75.

enzyme lost significant activity when the solution pH was adjusted to 9.0. The final preparation (lysophospholipase II) contained two main bands on SDS-PAGE.

Molecular Weight and Isoelectric Point of the P388D$_1$ Lysophospholipases—The purified lysophospholipase I has a molecular mass of 27,000 daltons as determined by SDS-PAGE. The molecular weight of lysophospholipase II was estimated by comparing the protein bands on SDS gels with the activities in each fraction from the Sephadex G-75 column; the peak at 28,000 correlated with the activity. Thus, the molecular weight of Blue II was estimated to be 28,000. The isoelectric point of lysophospholipase I is 4.4 and of lysophospholipase II is 6.1 as determined by isoelectric focusing chromatography (Fig. 4).

Stability—Enzyme stability was a major problem in the purification of this enzyme. After the DEAE column, the enzyme solution could not be stored at −20 °C in the absence of glycerol (see below) since the enzyme lost all of its activity during the freezing and thawing process. It was, however, possible to store the enzyme solution at 4 °C for several weeks with little loss of activity. The enzyme was unstable at pH values lower than 6.0 or higher than 8.5.

The purified enzyme was also extremely unstable. The enzyme lost half of its activity within 24 h and was completely inactive in 3 days. This instability was overcome by concentrating the enzyme solution in 100% glycerol. (See "Experimental Procedures"). The purified enzyme solution was usually concentrated to 10–25 μg ml^{-1}, and the final preparation contained 60–70% glycerol. With this treatment the enzyme retained its activity for at least several months at −20 °C. Dilution of the glycerol concentrated lysophospholipase I into aqueous buffer resulted in immediate loss of activity, but the enzyme is perfectly stable at room temperature for at least 24 h when it is diluted into 50% glycerol containing 10 μM BME. The enzyme did not lose activity when it was added directly into the assay mixture. Silanized vials were used for storing

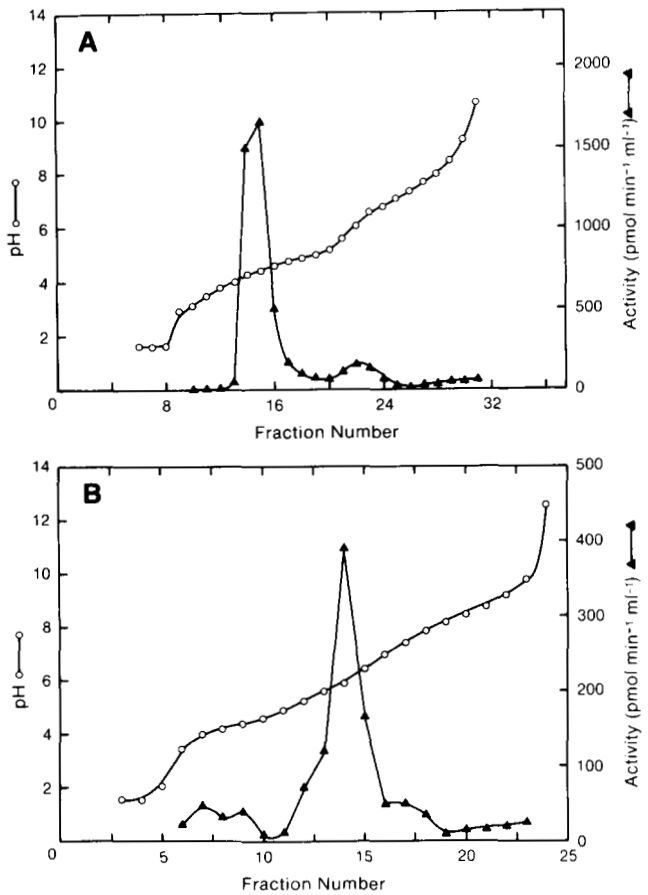

FIG. 4. **Isoelectric focusing of the two forms of lysophospholipase: *A*, lysophospholipase I and *B*, lysophospholipase II.**

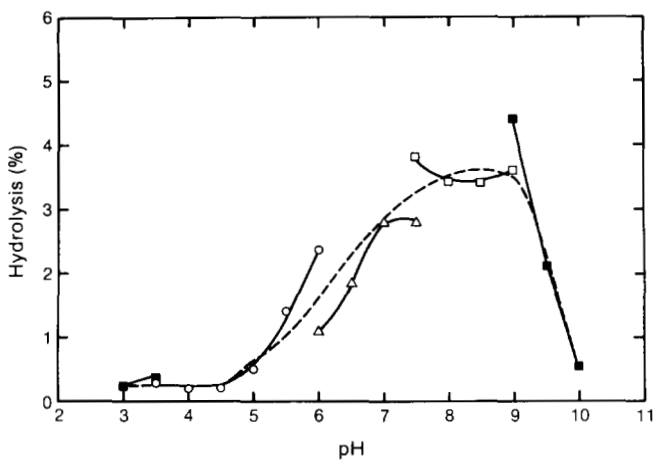

FIG. 5. **pH-rate profile of lysophospholipase I.** Standard assay conditions were employed except that 0.1 M NaCl and 0.1 M of the following buffers were present in the assay mixture: ○, acetate buffer; △, imidazole buffer; □, Tris-HCl buffer; ■, glycine buffer.

the enzyme solution since the enzyme tends to stick to glass or plastic and loses activity.

Kinetic Parameters—The hydrolysis of lyso-PC by 45 ng of purified lysophospholipase I was linear up to 45 min and 8% hydrolysis under standard assay conditions. Under these conditions the hydrolysis was linear with protein up to at least 160 ng. The pH optimum of the purified lysophospholipase was between pH 7.5 and 9.0 with some buffer effects as shown in Fig. 5. At pH values higher than 10, the nonenzymatic hydrolysis of lyso-PC was significant (28).

The substrate dependence of lysophospholipase I is shown in Fig. 6. When the data was plotted as a double-reciprocal plot (Fig. 6*B*), a break in two linear regions at about 10 μM was observed. The experiment has been repeated several times with different batches of purified enzyme. The break always occurred between 7–15 μM. An apparent K_m of 22 μM and V_{max} of 1.3 μmol min^{-1}mg^{-1} were obtained from the data between 15 and 200 μM lyso-PC.

Lysophospholipid substrate in micelles (above its CMC) was diluted by the addition of Triton X-100. Although the

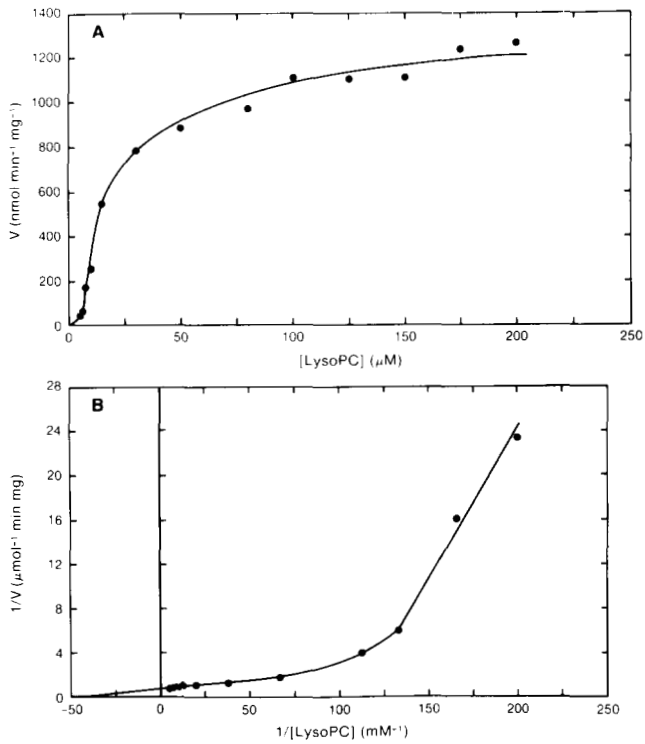

FIG. 6. ***A*, substrate dependence of lysophospholipase I and *B*, double-reciprocal plot of the data in A.**

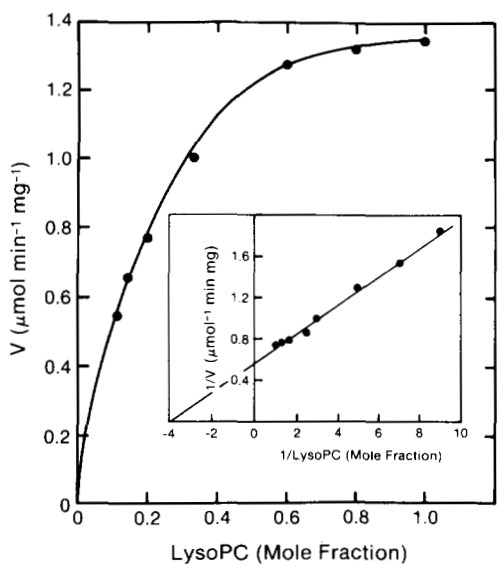

FIG. 7. **Dependence of lysophospholipase I activity on mol fraction of lyso-PC substrate in mixed micelles composed of lyso-PC and Triton X-100.** The mol fraction of lyso-PC is defined as [LysoPC]/[LysoPC] + [Triton X-100]. The bulk concentration of lyso-PC was kept constant at 150 μM and standard assay conditions were used. *Inset* is a double-reciprocal plot of the data.

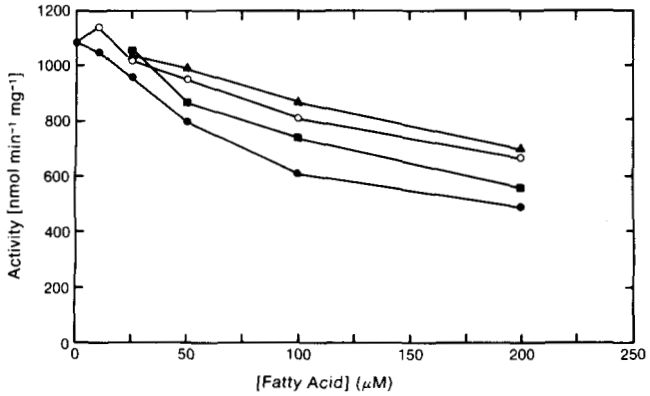

FIG. 8. **Inhibition of lysophospholipase I by fatty acids.** Standard assay conditions were employed except that fatty acid in ethanol (final concentration 2%) was added to each sample and control samples containing the same amount of ethanol (which is slightly inhibitory) but without fatty acid were subtracted: △, steric acid; ○, arachidonic acid, ■, oleic acid; and ●, palmitic acid.

TABLE II

Metal ion effect on lysophospholipase

Metal ion	Relative activity
10 mM	%
None	100
EDTA	104
Ca^{2+}	113
Mg^{2+}	106
Mn^{2+}	31
Fe^{2+}	94
Co^{2+}	4
Zn^{2+}	2
Cu^{2+}	1
Hg^{2+}	1
Fe^{3+}	63

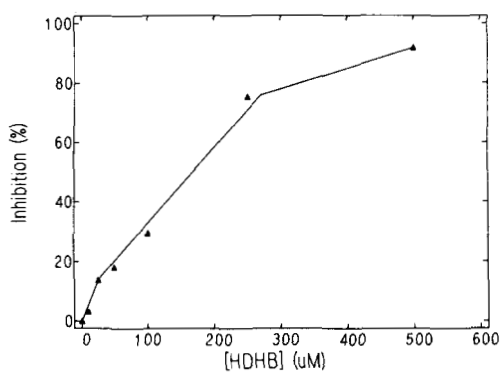

FIG. 9. **Inhibition of lysophospholipase I by HDHB.** Standard assay conditions were used except that 250 ng of lysophospholipase I was employed and HDHB dissolved in Me_2SO (10 μl) was added to samples. Control samples also contained 10 μl of Me_2SO.

Triton concentration was below its CMC, the monomers should be comicellized with the lysophospholipid micelles forming mixed micelles in which the lysophospholipid is diluted in the surface (29). Lysophospholipase I exhibits "surface dilution" kinetics in the presence of the mixed micelles as shown in Fig. 7. The double-reciprocal plot gave a V_{max} of about 1.8 μmol $min^{-1}mg^{-1}$ and a K_m of about 0.2 in mol fraction units.

Fatty Acid and Other Product Effects on Lysophospholipase I—Fatty acids had a small inhibitory effect on the purified lysophospholipase I which is shown in Fig. 8. The other product, glycerol phosphorylcholine, and related esters, glycerol phosphorylethanolamine, and glycerolphosphorylserine (50–200 μM), had no effect on the enzyme activity.

TABLE III

Related enzymatic activities in lysophospholipase preparations

All activities are expressed as nmol min^{-1} mg^{-1}.

Enzyme	Whole cell lysate	LS-1	Lysophospholipase I
Lysophospholipase	0.22	0.22	1700
Palmitoyl coenzyme A acyltransferase	0.50	0.13	0
Oleoyl coenzyme A acyltransferase	0.86	0.25	0
Transacylase	0.012	0	0
Phospholipase A_1	ND[a]	ND	0
Phospholipase A_2	0.034	ND	0

[a] Not determined.

Metal Ion Effects on Lysophospholipase I—Metal ion effects on the enzyme are shown in Table II. Divalent metal ions such as Ca^{2+}, Fe^{2+}, and Mg^{2+} had no effect on the enzyme activity while Hg^{2+}, Zn^{2+}, Cu^{2+}, and Co^{2+} inhibited the enzyme significantly. Trivalent Fe^{3+} ion partially inhibited the enzyme.

Inhibition of Lysophospholipase—HDHB is a synthetic manoalide analogue which has been shown to be an inhibitor of the phospholipase A_2 from cobra venom (19) as well as the phospholipase A_2 from the macrophage-like cell line P388D$_1$ (3). Fig. 9 shows that HDHB also inhibited the purified lysophospholipase I. Half-inhibition (IC_{50}) was found to be at about 160 μM. It did not appear to be due to irreversible inhibition since no inhibition was found when the lysophospholipase I was preincubated with 500 μM HDHB at 22 °C for 12 h and then assayed under standard assay conditions (HDHB was diluted to 5 μM in the assay mixture).

On the other hand, *para*-bromophenacylbromide (100 μM) which is a specific irreversible inhibitor of the cobra venom phospholipase A_2 (30) did not inhibit the lysophospholipase I in similar preincubation experiments. The carboxylesterase inhibitor bis-(*p*-nitrophenyl) phosphate also did not show inhibition of the lysophospholipase, although it has been reported to inhibit beef liver lysophospholipase I (17). When a serine protease inhibitor phenylmethylsulfonyl fluoride (1 mM) was incubated with the P388D$_1$ lysophospholipases in the assay mixture without preincubation, only minor inhibition was observed toward lysophospholipase I (11%) and lysophospholipase II (7%). However, when 2–5 mM phenylmethylsulfonyl fluoride was preincubated with the enzymes for 10 min at room temperature and then diluted into the assay mixture (final concentration 1 mM), significant inhibition was observed toward lysophospholipase I (87%) and lysophospholipase II (70%). Interestingly, phenylmethylsulfonyl fluoride has been reported to inhibit beef liver lysophospholipase I and II differently (17).

Specificity—The purified lysophospholipase I did not have phospholipase A_1, phospholipase A_2, acyltransferase, or transacylase activity. The relative activities of the latter two enzymes in the whole cell lysate and LS-1 are shown in Table III. No activity was detected toward triacylglycerol, diacylglycerol, *p*-nitrophenol acetate, *p*-nitrophenol palmitate, or cholesterol ester. However, the enzyme exhibited some activity toward monoacylglycerol The specific activity of the enzyme toward monoolein was 56 nmol $min^{-1}mg^{-1}$ or about 5% of its activity toward lyso-PC.

DISCUSSION

Lysophospholipase Purification and Stability—The instability of the enzyme was a major obstacle to developing an

adequate purification scheme. Some of the same problems were observed with the beef liver lysophospholipase I and the rabbit myocardium cytosolic lysophospholipase purifications (5, 17) which yielded preparations lacking the stability necessary for detailed kinetic studies. While the reason for the instability is still not very clear, two factors have been considered to contribute to it. The first is that a very small amount of enzyme is being purified and in dilute solution proteins tend to denature. The second factor is that this enzyme appears to have an essential SH group which is protected by BME. Because the presence of BME is essential for activity, it is difficult to test standard thiol reagents in the absence of BME. However, 1.0 mM 5, 5′-dithiobis-(2-nitrobenzoic acid) did partially inhibit lysophospholipase in the presence of 0.6 mM BME. Heavy metal ion inhibition and freezing/thawing denaturation phenomenon probably also relate to this property. Based on these considerations, BME and EDTA were generally included throughout the purification. For some steps where the BME or EDTA could not be used, such as Blue-Sepharose and the chromatofocusing chromatography, BME or EDTA was added back as the protein eluted from the column. Glycerol treatment was used as a final stabilizing step. The dramatic change of the enzyme's stability with this treatment argues that the enzyme benefits from a less polar environment.

Substrate appears to protect the enzyme activity. This has been shown by Gross and Sobel (5) in their thermal denaturation experiment. We have found that when the glycerol-stabilized enzyme solution was diluted into buffer (not containing glycerol), the enzyme lost activity immediately. However, adding enzyme solution to the assay mixture did not denature the enzyme, as shown by the linearity of the time course and the protein dependence measurement.

The molecular weight of the purified lysophospholipase I was determined by both gel filtration and SDS-PAGE. The agreement of the two measurements indicates that the enzyme is a monomer. The molecular mass of this enzyme (27,000 daltons) is similar to the beef liver lysophospholipase I and the rabbit myocardium cytosolic lysophospholipase which are 25,000 (17) and 23,000 (5) daltons, respectively. Both also appear to be monomeric enzymes.

Comparison of Lysophospholipase Forms—Two forms of lysophospholipase were found in the P388D$_1$ cells. They were completely separated by the Blue-Sepharose column. While the lysophospholipase I has been purified to homogeneity, the purification of lysophospholipase II has not been as successful on similar columns. The isoelectric points of these two forms are very different (4.4 for lysophospholipase I and 6.1 for lysophospholipase II), but their molecular mass is quite close (27,000 and 28,000 daltons). The structural homologies and the cellular locations of the two lysophospholipases in the P388D$_1$ cells need to be explored further. The existence of two forms of lysophospholipase has also been observed in *Escherichia coli* (31–33), beef liver (17), human ammion (4), rabbit myocardium (5), and germinating barley (34). Unfortunately, the forms of lysophospholipase from each source are not readily comparable.

Substrate Dependence—The double-reciprocal plot of the substrate dependence curve showed a break at about 10 μM palmitoyl lyso-PC. There are several possible causes of this abrupt change in enzyme activity. First, since the break is at the CMC of the substrate, which is around 7 μM (35–38), it could reflect a different affinity of the enzyme toward the monomeric and micellar substrate forms. This possibility needs to be further examined by studying enzyme activity toward different chain length lyso-PCs which have different CMCs. Second, since substrate protects the enzyme activity against irreversible inactivation, the extremely low enzyme activity toward low substrate concentrations could reflect the lack of protection. Third, the monomeric substrate at very low concentrations may not be accessible to the enzyme. These possibilities are presently under investigation in our laboratory.

The literature on lysophospholipase substrate-dependence kinetics is quite contradictory. Leibovitz-BenGershon *et al.* (39) reported that a 20-fold purified lysophospholipase from rat brain has activity toward monomeric lyso-PC but not the micellar form. De Jong *et al.* (40) tested beef pancreas lysophospholipase and beef liver lysophospholipase II with different chain length lyso-PC (containing from 6 to 18 carbons) and showed the enzymes do not have an abrupt velocity change at the CMC. Kawasawa *et al.* (33) also reported that lysophospholipase L2 from *E. coli* does not show any preference between monomeric or micellar forms of 2-acyl lysophosphorylethanolamine. Jarvis *et al.* (4) on the other hand, showed that the human amnionic lysophospholipase exhibited similar kinetics to those reported here for the P388D$_1$ lysophospholipase I in that it exhibited a break in the double-reciprocal plot at 6–7 μM palmitoyl lyso-PC.

It has been reported that Triton X-100 inhibits lysophospholipase activity (34, 41, 42). When the Triton effect was tested on the P388D$_1$ lysophospholipase I, the enzyme activity decreased with increasing Triton X-100 concentrations. However, this phenomena could be caused by surface dilution (43) of the substrate in the mixed micelles. Lysophospholipase activity toward the lyso-PC/Triton X-100 mixed micelles was dependent on the lyso-PC mol fraction. The linearity of the 1/v *versus* 1/lyso-PC mol fraction curve indicates that this decrease in activity does follow the dilution of the substrates in the surface. This is similar to observations made with soluble phospholipases (44).

Inhibition of Lysophospholipase I—The purified lysophospholipase I was found to be inhibited by its fatty acid product. Although the inhibition was not very strong, it could be one of the mechanisms by which the cells control the lysophospholipid metabolic pathways. A synthetic manoalide analogue, HDHB, was also found to inhibit the purified lysophospholipase I. The inhibition appeared to be similar to that of phospholipase A$_2$ from P388D$_1$ cells (3), and from cobra venom (19), although the IC$_{50}$ toward the lysophospholipase was higher (160 μM) than that toward the phospholipase (40 μM) from the macrophage. Since the inhibition was reversible toward all three enzymes, it was possible that the lysophospholipase I has a similar active site to the phospholipase A$_2$s. However, *p*-bromophenacyl bromide, a chemical modification reagent, which inhibits phospholipase A$_2$ from cobra venom completely (30) did not inhibit the lysophospholipase I at all under the experimental conditions employed.

Metabolic Role of Lysophospholipase—There are many interesting reports suggesting that the accumulation of lysophospholipids causes cellular structural or functional change (1). It is generally assumed that the accumulation of lysophospholipids is caused by phospholipase A activation. However, the concentration of lysophospholipids is determined by at least five different enzymes. Since active turnover of phospholipids occurs (45), any inhibition of the lysophospholipid-removing enzymes, which usually have a much higher activity than the lysophospholipid-producing enzymes, could equally as well be the cause of lysophospholipid accumulation. We hope that the availability of the pure lysophospholipase and the pure phospholipase A$_2$ and their characterization will now allow us to proceed with an investigation of the details of the

regulation of lysophospholipid metabolism in intact cells.

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