Effects of acidic phospholipids, nucleotides, and heparin on the activity of lipase from rat liver lysosomes

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Abstract Purification and characterization of endogenous lipid factors that stimulate rat liver lysosomal lipase has led to the identification of cardiolipin, phosphatidylserine, and phosphatidic acid as stimulators of this activity. Bovine heart cardiolipin (half-maximal stimulation at 1.5×10^{-4} M) and bovine brain phosphatidylserine (half-maximal stimulation at 9.5×10^{-4} M) were the most potent of the phospholipids from other sources tested. The major rate-enhancing effect of phosphatidylserine is expressed as a 35-fold increase in the apparent V_{max} of the enzyme. The effect is produced by acid phospholipids specifically, since in no case was there greater than a two-fold stimulation by synthetic detergents, zwitterionic phospholipids, taurocholic acid, or gum acacia. The observed degree of stimulation depends upon the detergent used to disperse tripalmitin substrate and the relative concentrations of factor and detergent in reaction mixtures. The concentration of phosphatidylserine to produce half-maximal stimulation is directly dependent upon the Triton X-100 concentration, but the effects of this detergent on cardiolipin stimulation are more complex. Enzyme activity is inhibited 50% by 1 mM nucleoside triphosphate and 2.5 mM ADP, 80% by 1 mM PP_i, 100% by 20 U/ml heparin and 0.25 mg/ml chondroitin sulfate, and 80% by 10 mM sulfate ion. Inhibition is partially prevented by phosphatidylserine.

Supplementary key words cardiolipin · phosphatidylserine

Lysosomes play a role in degradative processes including those associated with the induction of antibody synthesis (1), viropexis (2), and the turnover of intracellular metabolites (3). Most lysosomal enzymes are hydrolytic and exhibit acidic pH optima (4). The lysosomes in rat liver contain an acid lipase (5, 6) which may be stimulated by glucagon in vivo (7). Lysosomal lipase may be defective in Wolman's syndrome (8).

The degree of purification reported for acid lipase in lysosomal fractions is sometimes less than that reported for acid phosphatase (9). Hayase and Tappel (5) found that 80% of the enzyme activity was lost during chromatography on hydroxylapatite columns, and other workers (10) could not detect the activity in purified tritosomes. Problems of this sort, which suggest that stimulatory factors are needed for enzyme activity (5), have hindered efforts to purify the enzyme. We recently reported direct evidence for the presence in rat liver homogenates of lipids that stimulate activity (11).

Phospholipids can stimulate activity either by perturbing the dispersion form of insoluble substrate (12) or by interacting directly with enzymes (13–15). However, in most cases the exact mechanism of the effects are poorly understood (16–19).

We decided to investigate in detail the nature of the phospholipid stimulation of rat liver lysosomal lipase. Of the phospholipids studied, cardiolipin was the most efficient endogenous stimulator of the lipase. Studies also demonstrate rate-enhancing properties associated with acidic phospholipids, including a 35-fold increase in the V_{max} of the enzyme, the prevention of apparent substrate inhibition at low octylphenoxypolyethoxyethanol (Triton X-100)-to-substrate ratios, and partial prevention of inhibition of the enzyme by nucleotides, sulfated polysaccharides, and other anions.

MATERIALS AND METHODS

Materials

Glyceryl tri[9,10-$^3H_2$]palmitate was obtained from Amersham/Searle (Des Plaines, Ill.). Lysolecithin (from egg lecithin), phosphatidylserine (brain extract, type III), and unlabeled tripalmitin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Beef heart cardiolipin, phosphatidylinositol (plant), phosphatidylethanolamine (bovine), and phosphatidic acid were obtained from Applied Science Laboratories (State College, Pa.). The purity of tripalmitin and the phospholipids was confirmed by thin-layer chromatography. Triton X-100,

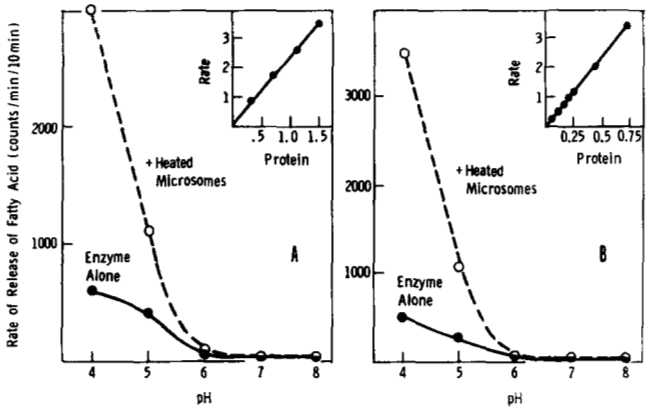

FIG. 1. Effect of pH on lipase activity in the presence and absence of endogenous factors, (*A*) soluble lipase preparation routinely used in these studies and (*B*) lipase extracted from purified lysosomes. The tripalmitin concentration was 1 or 2 mM, respectively, for measurement of the pH–activity relationships of basal or lysosomal enzyme. Values below pH 4 could not be accurately measured because the enzyme is rapidly inactivated at these pH values. The rates in the insets are expressed as neq/min.

ATP, UTP, CTP, GTP, ADP, AMP, EGTA (ethylene glycol-bis[β-aminoethyl ether]tetraacetic acid), heparin (170 units/mg), sodium lauryl sulfate, taurocholic acid, and chondroitin sulfate were purchased from Sigma Chemical Co. EDTA, 1,10-orthophenanthroline, and gum acacia were purchased from Pierce Chemical Co. (Rockford, Ill.). *N,N*-Dimethyldodecylamine was from K & K Laboratories, Hollywood, Calif., and Sarkosyl NL-30 was obtained from Geigy Chemical Corp., Ardsley, N.Y. Silicic acid (lipid chromatography grade, Sigma Chemical Co.), Hyflo Supercel (Fisher Chemical Co., St. Louis, Mo.), and precoated silica gel G plates (Analtech, Inc., Wilmington, Del.) were also used. Male Sprague-Dawley rats (150–250 g) were from Carworth Farms (Portage, Mich.).

Enzyme and factor preparations

The solubilized enzyme used in these studies was prepared from the 0–12,000 *g* particulate fraction of rat liver homogenates (11) by extraction (5) with 0.05 M sodium phosphate buffer, pH 6.8. Rat liver microsomes and lysosomes obtained by the procedure of Ragab et al. (20) were, respectively, the source of heat-stable factor and a 20-fold purified lipase preparation. Microsomes were heated at 70°C in 0.05 M sodium phosphate buffer, pH 7.0, for 15 min to obtain preparations of factor free of lipase activity.

Measurement of lipase activity

Tripalmitin dispersions in Triton X-100 were prepared by the method of Kaplan and Teng (21). Phospholipids were sonicated in distilled water or in 0.5% Triton X-100 for 30 sec (microprobe intensity of 70%) at 4°C with a Biosonic II sonicator.

The following standard reaction conditions were used unless otherwise indicated: 20 mg of Triton X-100, 50 μmoles of sodium acetate buffer, pH 4, and 1 μmole of [^{3}H]tripalmitin (either 1.37 or 1.64 × 10^6 dpm/μmole) in 0.7 ml were incubated at 37°C for 3–6 hr before the addition of enzyme, phospholipid, other reagents, and water to bring the final volume to 1.0 ml. Reactions were stopped after 10 min at 37°C by adding 5 ml of isopropanol–heptane–1 N sulfuric acid 40:10:1 (by vol). Free fatty acid release was assessed according to Kaplan (22) by the measurement of heptane-soluble radioactivity extractable by alkaline ethylene glycol. The range of duplicate analyses never exceeded ± 3%. Zero-time values in the presence and absence of acidic phospholipids were identical. The addition of acidic phospholipids, after reactions were stopped, had no effect on the recovery of released fatty acid. Proteins were assayed by the procedure of Lowry et al. (23).

Preparation of rat liver lipid extracts and silicic acid for column chromatography

Rat liver (14 g wet wt) was homogenized in a Waring blender for 30 sec at high speed at 4°C with 42 ml of 50 mM magnesium acetate in 0.25 M sucrose (pH 7.0). The homogenate was subjected to centrifugation for 90 min at 100,000 *g*. The pellet was mixed on a magnetic stirrer with 50 ml of ethanol–ethyl ether 3:1 (v/v) for 6 hr and the suspension was centrifuged at 11,000 *g* for 20 min.

Remaining lipids in the residues were extracted three times with chloroform–methanol 2:1 (v/v) by the procedure described above. The supernatant fluids obtained from all extractions were pooled and evaporated to dryness under a nitrogen stream.

Silicic acid was heated at 115°C for 4 hr, mixed with Hyflo Supercel at a ratio of 2:1, and washed three times by stirring for 20 min with methanol, which was then removed by decantation. Columns (1.5 × 30 cm) were prepared from this material and conditioned by treatment with 3 column volumes of chloroform. Phospholipid phosphorus was determined by the method of Shimojo, Kanoh, and Ohno (24).

RESULTS

Stimulation of lysosomal lipase by endogenous factors

These studies were performed with preparations of solubilized lysosomal lipase which contained 25% of the acid lipase activity and 3.5% of the protein of rat liver homogenates but no neutral or alkaline lipase activity. The acid lipase activity was stimulated by endogenous factors without alteration of the pH optimum, and the enzyme exhibited a linear activity vs. protein concentration relationship indicative of the absence of dissociable activators. The rate of hydrolysis of the triglyceride in tripalmitin–Triton X-100 dispersions was constant for

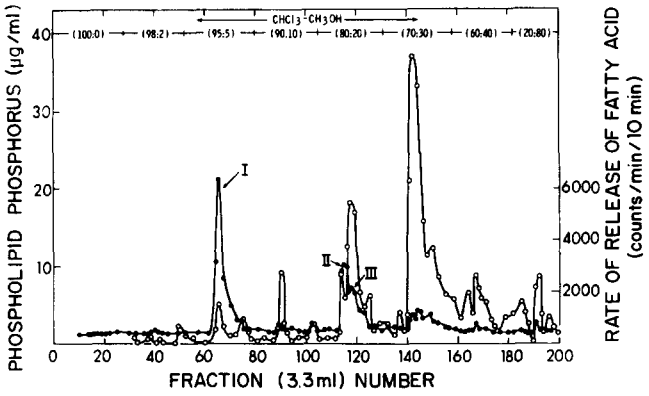

FIG. 2. Separation of stimulatory factors in rat liver lipids by silicic acid column chromatography. The column (prepared as described in Methods) was charged with 270 mg of rat liver lipids dissolved in 12 ml of chloroform. The flow rate was 0.3 ml/min. The stimulatory activity of each fraction was determined after the addition of the solids obtained from 1-ml aliquots to standard reaction mixtures containing 0.8 mg of basal enzyme. (○) organic phosphate; (●) lipase activity in the presence of the fraction solids.

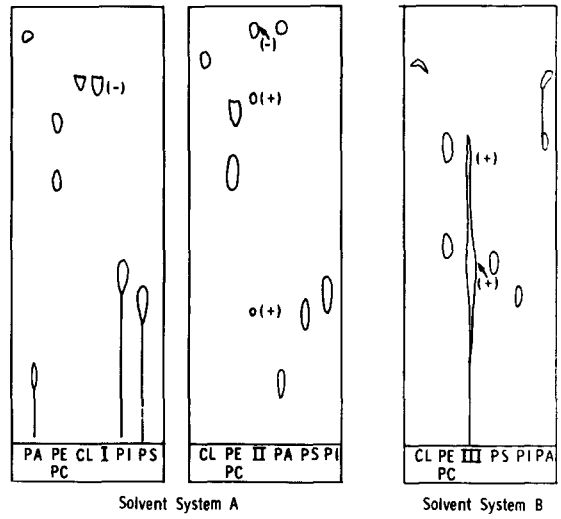

FIG. 3. Thin-layer chromatograms of components in peaks I, II, and III obtained from silicic acid column chromatography of rat liver lipids. Solvent system A was chloroform–methanol–7 N NH_4OH 12:7:1 (v/v/v). Solvent system B was chloroform–acetone–methanol–acetic acid–water 50:20:15:10:6 (v/v/v). After development of the plates, which contained 20–40-μg aliquots of the fraction of each peak containing maximum activity, spots were visualized by staining for phospholipid phosphorus or by reaction with ninhydrin. The results of the ninhydrin tests are indicated by the bracketed sign next to each experimental phosphate-positive spot. PA is phosphatidic acid, PE is phosphatidylethanolamine, PC is phosphatidylcholine, CL is cardiolipin, PI is phosphatidylinositol, and PS is phosphatidylserine.

the first 10 min of reaction. Lipase of greater purity, extracted from lysosomes prepared by the procedure of Ragab et al. (20), behaved in an identical fashion (Fig. 1).

Characterization of factors

The endogenous lipid stimulators have been identified as acidic phospholipids by procedures summarized in Figs. 2 and 3. When lipid from the particulate fraction of rat liver homogenates was chromatographed on silicic acid columns (Fig. 2), three peaks of stimulatory components were observed. Thin-layer chromatography (Fig. 3) of aliquots of the fraction of peak I, which contained the most stimulatory activity, revealed a single phosphate-containing compound which had the same relative mobility and negative response to ninhydrin as cardiolipin in both solvent systems A and B. By these criteria, peak II contained a mixture of phosphatidic acid and phosphatidylserine. (Because of the poor separation of phosphatidic acid and cardiolipin in solvent system B, only the results for peaks I and II from solvent system A are presented in Fig. 3.) Peak III contained a mixture of phosphatidylserine and phosphatidylethanolamine. Stimulation by the components of this peak is probably due to the trailing shoulder of phosphatidylserine. The peak I component, tentatively identified as cardiolipin, was found to be the most efficient lipid stimulator of lysosomal lipase (Fig. 2).

Effect of acidic phospholipid and Triton X-100 concentration on stimulation

The relationships of commercial beef heart cardiolipin and brain phosphatidylserine concentrations to enzyme activity at specified Triton X-100 concentrations were obtained as shown in Fig. 4. At the standard assay conditions (20 mg/ml Triton X-100), the concentration of phosphatidylserine required for half-maximal stimulation (K_{50} = 0.95 mM)[1] was six times greater than that of cardiolipin (K_{50} = 0.15 mM).[1] The K_{50} for phosphatidylserine is directly dependent upon the Triton X-100 concentration, and at low detergent concentrations the saturation curves for this phospholipid are characterized by optima. The concentration dependence of enzyme stimulation upon cardiolipin is more complex than that for phosphatidylserine.

Effect of substrate dispersing agent

Stimulation by phosphatidylserine was observed in the absence of Triton X-100 with substrates dispersed in gum acacia, taurocholic acid, or lysolecithin (Fig. 5) and with Sarkosyl NL-30 (not shown). At high concentration, taurocholic acid, gum acacia, and lysolecithin differed from Triton X-100 by inhibiting lipase activity in the absence of phosphatidylserine. All four agents inhibited lipase activity in the presence of this phospholipid.

[1] Molecular weights of 780 for brain phosphatidylserine and 1450 for beef heart cardiolipin were assumed.

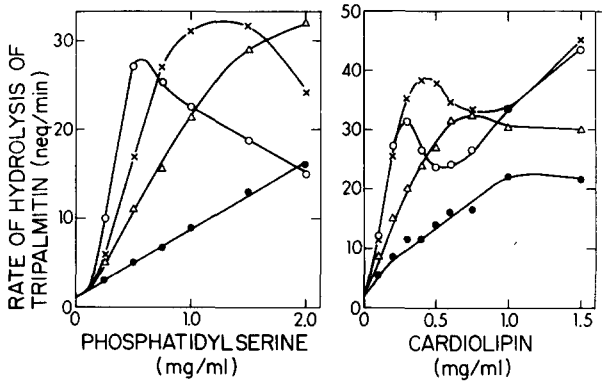

FIG. 4. Relationships of phosphatidylserine and cardiolipin concentrations to lipase activity. Basal enzyme (1.28 mg) was incubated under standard reaction conditions with the indicated amounts of phospholipid except that the Triton X-100 concentration (mg/ml) was 5 (○), 10 (×), 20 (△), or 40 (●).

Specificity of stimulation

The specificity of stimulation by phospholipids was also assessed. Cardiolipin, phosphatidylinositol, and phosphatidylserine produced equal degrees of stimulation (approximately 20-fold) at standard reaction conditions. As noted in Fig. 4 and in a previous report (11), considerable differences in the K_{50} for the various acidic phospholipids were detected. Zwitterionic phospholipids produced only meager stimulation comparable to acidic phospholipids (Fig. 6). The small degree of stimulation observed with Sarkosyl NL-30, with taurocholic acid, and with low concentrations of sodium lauryl sulfate was comparable to that observed with zwitterionic phospholipids. *N*,*N*-Dimethyldodecylamine and Tween 80 (not shown in Fig. 6) were inhibitory at all concentrations. Neither D-phosphoserine nor glycerophosphorylserine had any effect on the activity in the presence or absence of phosphatidylserine, indicating that the lipophilic fatty acyl moieties of the phospholipid are probably involved in the stimulation.

Effect of substrate concentration

The effect of substrate concentration on the stimulation of lipase by phosphatidylserine is presented in Fig. 7 and summarized in Table 1. At 30 mg of Triton X-100/ml, the major rate-enhancing effect of phosphatidylserine was observed as a 35-fold increase in the apparent V_{max} of the enzyme. Apparent inhibition observed with 2–3 mM tripalmitin was not seen in the presence of phosphatidylserine. One interpretation of this apparent inhibition is that it is due to a kinetically unfavorable aggregation of the substrate dispersion particles (21). From comparisons of kinetic parameters of the enzyme measured at two concentrations of Triton X-100 (Table 1), it can be seen that Triton X-100 itself has no effect on the apparent V_{max} of the enzyme but does increase the apparent K_m for substrate as does phosphatidylserine.

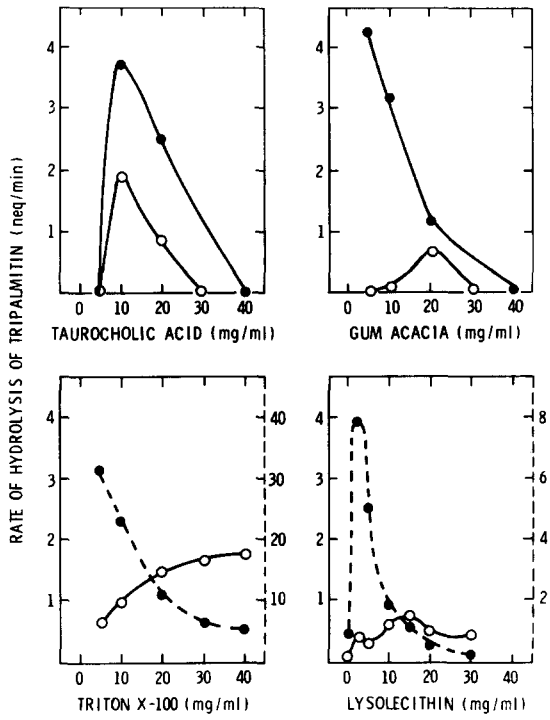

FIG. 5. Effect of detergent concentration and phosphatidylserine on lysosomal lipase activity with tripalmitin dispersed in taurocholic acid, gum acacia, Triton X-100, or lysolecithin as substrate. The dispersions of tripalmitin with detergents other than Triton X-100 were prepared by sonication at microprobe intensity of 60% for 30 sec of 1 μmole of tripalmitin with sufficient detergent in 0.8 ml of 62.5 mM acetate buffer (pH 4) to obtain the detergent concentrations in reaction mixtures indicated. Except for the replacement of Triton X-100 with other detergents, standard reaction conditions were employed. (●——●) indicates the activity measured in the presence of phosphatidylserine as indicated on the left ordinates, and (●- - - -●) indicates that activity as indicated on the condensed scales of the right ordinates. (○——○) is the activity meaured in the absence of phosphatidylserine.

Anion effects

Many anions were found to inhibit the basal enzyme activity. Table 2 summarizes the effects of nucleoside triphosphates and related anions on the activity in the presence and absence of phosphatidylserine. Nucleotides that contain at least two phosphoryl moieties were effective inhibitors. 1 mM nucleoside triphosphates inhibited 50% of the activity, as did 2.5 mM ADP. 2.5 mM AMP inhibited only 8%, and 5 mM P_i was essentially ineffective. PP_i inhibited dramatically at concentrations comparable to nucleoside triphosphates. These effects were partially prevented in the presence of 0.6 mg/ml phosphatidylserine. Only 10% inhibition by 1 mM ATP was observed at 1.5 mg/ml phospholipid. The inhibition by SO_4^{2-} and heparin is similarly prevented by phosphatidylserine (Fig. 8). In other experiments, chondroitin sulfate was also found to inhibit the enzyme activity.

EDTA, EGTA, and citrate were found to be poor inhibitors by comparison with PP_i (Table 3). Ortho- and

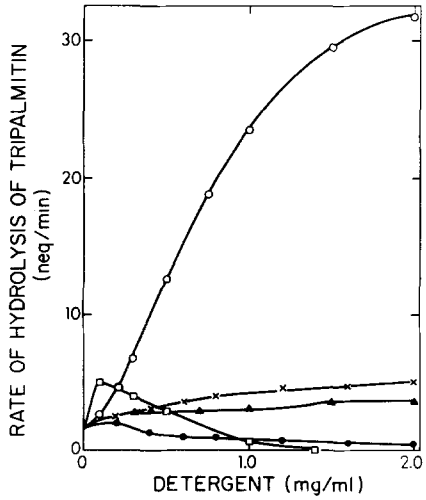

FIG. 6. Effect of detergent concentration on lipase activity with tripalmitin dispersed in Triton X-100 as substrate. The detergents employed were (O) phosphatidylserine, (□) sodium lauryl sulfate, (×) Sarkosyl NL-30, (●) *N*,*N*-dimethyldodecylamine, or (▲) taurocholic acid.

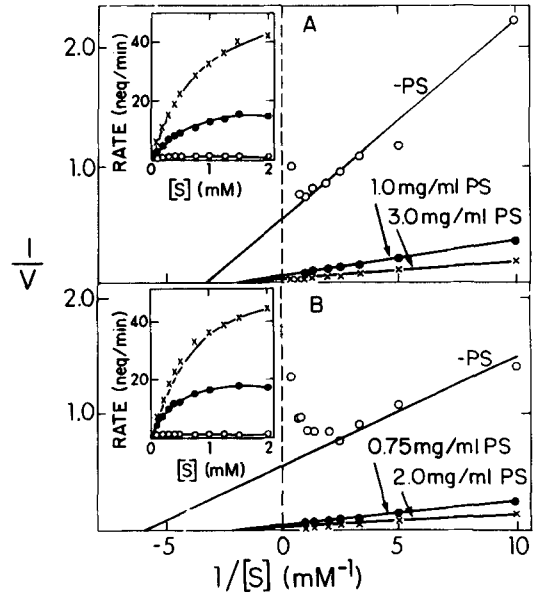

FIG. 7. Effect of phosphatidylserine on the relationship of tripalmitin concentration to lipase activity in the presence of Triton, *A*, 30 mg/ml, and *B*, 20 mg/ml. The lipase concentration was 0.85 mg/ml. PS is phosphatidylserine.

bathophenanthroline and dihydroxy benzene disulfonic acid exhibited no significant inhibition. All activity was retained after treatment of enzyme with 5 mM EDTA, EGTA, or PP_i at neutral pH and dialysis against buffers containing 50 μM EDTA. The activity of the treated and dialyzed preparations was unaffected by Ca^{2+}, Mg^{2+}, Na^+, or K^+. Phosphatidylserine depleted of divalent metal ions was a potent stimulator of the activity of the chelator-treated enzyme preparations. The data on fluoride in Table 3 confirms the findings of Hayase and Tappel (5) that the monovalent anion is an inhibitor of the enzyme. This inhibition is also depressed in the presence of phosphatidylserine.

DISCUSSION

Acidic phospholipids, and particularly cardiolipin, from rat liver and other sources stimulate rat liver lysosomal lipase activity. We have avoided the term "activation" when referring to these effects since it connotes direct modulation of enzyme by a modifier. None of the properties of the stimulation preclude activation. Indeed, with enzymes that catalyze the conversion of soluble substrates, such as *E. coli* pyruvate oxidase (13) and the Na^+-, K^+-dependent ATPase (14), activation seems to be the only explanation of the stimulation by phospholipids. However, if the substrate is insoluble, as in the case of tripalmitin, other possibilities exist, such as the kinetically favorable alteration of substrate dispersion forms by phospholipids. Such alterations have been postulated as explanations of the behavior of a solubilized beef liver lipase (21). The substrate specificity of phospholipase A, which catalyzes the hydrolysis of dioctanoylphosphatidylcholine at a 60 times greater maximal rate than that of the diheptanoyl or dinonanoyl derivatives, has been rationalized on the basis of structural differences among the dispersion forms of these compounds (25). Data related to the stimulation of this enzyme by acidic phospholipids have also been interpreted in this manner (12).

Lipid-mediated stimulation of lysosomal lipase appears to be restricted to acidic phospholipids. Neither zwitterionic phospholipids nor anionic detergents, including taurocholic acid, exhibit this effect. Stimulation was observed with a variety of initial dispersion forms of substrate. The major effect of the lipid is expressed as the enhancement of the maximum velocity of the enzyme. These findings may be useful in the development of models for the explanation of lipase stimulation. Since

TABLE 1. Influence of Triton X-100 and phosphatidylserine on kinetic parameters of lysosomal lipase with tripalmitin–Triton X-100 dispersions as substrate[a]

Triton X-100	Phosphatidylserine	K_m	V_{max}
mg/ml	*mg/ml*	*mM*	*neq/min*
20	0	0.16	1.8
	0.75	0.53	26.3
	2.0	0.71	62.5
30	0	0.29	1.8
	1.0	0.69	21.7
	3.5	0.91	62.5

[a] Assay conditions are described in the legend to Fig. 7. K_m and V_{max} values were obtained by extrapolation of the straight line portions of double reciprocal plots.

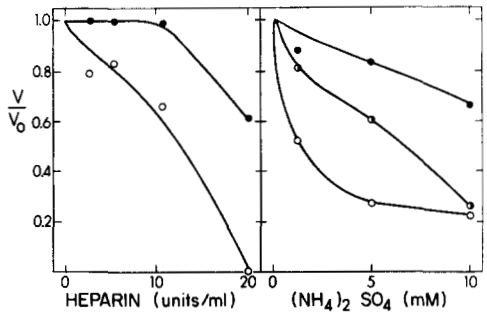

FIG. 8. Effect of heparin and SO_4 concentration on lysosomal lipase activity. Activity measured in the absence of phosphatidylserine (○), in the presence of 0.6 mg/ml phosphatidylserine (◑), and in the presence of 1.5 mg/ml phosphatidylserine (●). V_0 is the rate of hydrolysis in the absence of sulfate derivative and V is that rate in the presence of the indicated amount of derivative.

the enzyme is far from pure, the possibility exists that the factors may be acting via other proteins. This possibility precludes meaningful interpretation of binding studies with this preparation. In this regard, we have recently observed that the enzyme can be selectively eluted from carboxymethyl cellulose columns by phosphatidylserine liposomes and by mixtures of this phospholipid and Triton, but not by triglyceride–Triton mixtures. However, it seems prudent to refrain from interpretation of the data in mechanistic terms until experiments with purified enzyme and substrates with simpler phase structure are performed.

Many anions have been found to inhibit the activity of the enzyme. The degree of inhibition is greater in the absence of anionic phospholipids than in their presence. This may indicate a common enzyme site for these compounds. Further evidence for such a site can be obtained from binding studies. Although some of the inhibitory anions are chelators, the absence of any detectable effects of metal ions either before or after treatment of the enzyme with chelators at neutral pH is not in consonance with the proposal that the inhibition is caused by chelation of essential metal ions.

Nucleotides and heparin have been suggested as physiological modifiers of lipases (26, 27). The effect of modifiers reported in this paper cannot now be extrapolated to in vivo situations, since under those conditions the enzyme is located within the unknown microenvironment of the lysosome, and the substrate dispersion form must be different from that prepared in the laboratory.

These studies demonstrate many of the pitfalls that prevent the facile interpretation of quantitative aspects of lysosomal lipase activity in crude homogenates and the simple comparison of measurements of this activity with different substrate dispersion forms. For example, the reported increase in the specific activity of the lysosomal lipase of homogenate of rat livers after perfusion with glucagon might reflect a change in the concentration of cardiolipin mediated by glucagon (7). The inability of Franson, Waite, and LaVia (10) to detect acid lipase in rat liver tritosomes might be explained by their omis-

TABLE 2. Inhibition of lysosomal lipase by nucleoside triphosphates, ADP, and PP_i in the presence and absence of phosphatidylserine[a]

Compounds	Concn.	Inhibition by Compounds	
		Without PS[b]	With PS[b]
	mM	%	
CTP	2.0	52	
	1.0	51	
	0.5	31	
GTP	2.0	61	
	1.0	54	
	0.5	52	
UTP	2.0	58	
	1.0	55	
	0.5	47	
ATP	2.0	68	58
	0.5	51	35
PP_i	5.0	84	63
	1.0	78	28
ADP	2.5	49	18
	1.0	35	11
	0.5	14	3
AMP	5.0	14	17
	2.5	8	8
	1.0	0	4
P_i	10.0	16	21
	5.0	5	15
	1.0	0	6

[a] After basal enzyme was dialyzed for 24 hr against 0.02 M ammonium acetate, aliquots containing 0.85 mg were incubated in the presence or absence of 0.6 mg/ml phosphatidylserine under the standard reaction conditions except that the indicated amounts of the sodium salts of the inhibitors were added.

[b] PS, phosphatidylserine.

TABLE 3. Inhibition of lysosomal lipase by chelating anions and fluoride in the presence and absence of phosphatidylserine[a]

Compounds	Concn.	Inhibition by Compounds	
		Without PS[b]	With PS[b]
	mM	%	
EDTA	10.0	51	47
	5.0	40	29
	1.0	20	13
EGTA	5.0	48	30
	1.0	38	0
Citrate	10.0	39	10
	5.0	32	0
	1.0	9	0
F	5.0	100	91
	1.0	84	67
	0.5	76	47
Cl	5.0	0	
	1.0	0	8

[a] Reaction conditions were identical with those described for Table 2, except that the effects of NaF were tested with undialyzed basal enzyme. Sodium salts of all anions were used.

[b] PS, phosphatidylserine.

sion of acidic phospholipids from reaction mixtures. The anomolies associated with the lack of identical distribution of acid lipase and acid phosphatase among the subfractions obtained from differential centrifugation procedures for the purification of lysosomes (9) can be rationalized in the light of the differential distribution of acid lipase and endogenous acidic phospholipids. We have been able to assay acid lipase activity as a linear function of protein concentration even in crude homogenates by the addition of 1.5 mg/ml of phosphatidylserine to standard reaction mixtures. When this modification was employed, the distribution of the lipase activity among the subfractions obtained by the procedure of Ragab et al. (20) was found to be identical with that reported for acid phosphatase.

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