EVIDENCE OF A MEMBRANE-BOUND PHOSPHOLIPASE A IN RAT LIVER LYSOSOMES

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Received December 8, 1969

Summary

A phospholipase A with optimal activity at pH 7.0-8.0 was found in the membranes of rat liver lysosomes. This enzyme is activated by Ca^{++}, and is specific for the fatty acid at the 2-position. The presence of this enzyme in lysosomes is not due to mitochondrial contamination.

Introduction

Phospholipid-degrading enzymes have been reported to be present in lysosomes of various tissues (Gatt *et al*, 1966; Elsbach, 1966; Blaschko *et al*., 1967; Mellors *et al*, 1967; Fowler & de Duve, 1969; Stoffel & Trabert, 1969). Mellors *et al*, (1967) reported an acid phospholipase (pH 4.5) in rat liver lysosomes that was located mainly in the membranes of these particles; but in a later report (Mellors & Tappel, 1967) showed, contrary to their previous study, that the acid phospholipase activities can be recovered almost entirely in the soluble fraction obtained from the lysosomes.

Our recent observations presented here indicate acid phospholipase is indeed localized in the soluble fraction of the lysosomes; unexpectedly a phospholipase with optimal activity at pH 7.5 was also obtained from the membrane fraction of these particles. This phospholipase in the lysosomal membranes was not due to mitochondrial contamination.

Methods

Synthesis of Substrate. 1-Palmitoyl-2-^{14}C-linoleoyl-glyceryl-3 phosphorylethanolamine was the substrate for these phospholipase A determinations. It was synthesized according to procedure described by van den Bosch *et al*, (1968). By subsequent hydrolysis of the

substrate with snake venom phospholipase A, 97% of the ^{14}C-linoleic acid was demonstrated to be in the 2-position of the ^{14}C-phosphatidyl ethanolamine. This substrate had a specific activity of 1.96×10^9 disint. per min. per mmole.

Determination of Phospholipase A Activities. Approximately 20 nano moles of ^{14}C-phosphatidyl ethanolamine were emulsified by a brief ultrasonication in 1 ml of Tris-maleate buffer (0.1M) of varying pH values. Isolated lysosomes or mitochondria, with 100 µg of protein content were then added to a final volume of 1.5 ml, in the presence (or absence) of 8mM $CaCl_2$. After incubation at 37°C for 30 minutes, lipids were extracted directly by the method of Bligh & Dyer (1959), followed by two additional extractions with chloroform. A small amount of a mixture, containing nonradioactive phosphatidyl ethanolamine, lyso-phosphatidyl ethanolamine, and free fatty acids, was added to the lipid-containing layer; which was then dried and redissolved in 10 drops of methanol-chloroform mixture. The total volume of this mixture was analyzed by chromatography. Thin layer chromatography was used to separate the remaining phosphatidyl ethanolamine (PE) and its hydrolysis products, i.e., lyso-phosphatidyl ethanolamine (LPE) and free fatty acids (FFA), with a chloroform-methanol-water (65:35:4 by vol.) solvent system. After development of the chromatograms, the compounds were outlined under I_2 vapor and the separated spots (PE, LPE and FFA) were quantitatively scraped from the chromatographic plates, and were transferred directly to scintillation vials containing the dioxane scintillation mixture. Radioactivity was determined in a Packard Tri-Carb liquid scintillation counter. The radioactivities found from the FFA spot, plus that from the LPE spot, represent the total hydrolysis in each reaction.

The phospholipase activities are expressed either as nano moles or as percentage of substrate hydrolyzed after the indicated period of incubation. Blanks obtained from incubations were subtracted from the total activity in all determinations for each experiment.

Determinations of Protein and Marker Enzymes. Protein concentration was determined by Lowry's method (1951). Acid phosphatase, cytochrome oxidase and glucose 6-phosphatase activities were assayed according to de Duve *et al*, (1955). Urate oxidase was determined according to Beaufay *et al*, (1959).

Preparations of Subcellular Fractions. Wistar rats of either sex were used throughout these experiments. Composite fractions of mitochondria and lysosomes (M+L) were prepared according to Appelmans and de Duve (1955).

Mitochondria and lysosomes were prepared from rats injected with Triton WR-1339 (0.85 g per kg. of body weight) (Wattiaux *et al*, 1963).

Mitochondria were prepared as follows: nuclei were sedimented from a 10% (w/v) liver homogenate at 1,000x g for 10 minutes in an International centrifuge PR-6 with no 269 swinging bucket rotor; the supernatant was then centrifuged at 9,000 x g for 10 minutes in a Servall centrifuge to obtain a crude mitochondrial fraction. Resuspended, crude mitochondria were layered on top of a 40 ml continuous sucrose gradient which consisted of five successive layers of sucrose of the following percent concentrations: 25, 30, 35, 40, and 45%. The volume of the first three layers was 5 ml each, that of the fourth layer was 15 ml, and the bottom layer was 10 ml. Each sucrose gradient was prepared manually with a bent needle and was stored in the cold room ($0^{o}C$) overnight. Then, the gradients were centrifuged at 3,500 x g for two hours in the International centrifuge PR-6. The purified mitochondria were collected at the 40%-sucrose layer. The mitochondria were minimally contaminated by lysosomes and microsomes, as ascertained by acid phosphatase and glucose-6-phosphatase activities. In no case did these exceed 2% when compared to their respective activities in the homogenate. Lysosomes were prepared by the floatation method described by Trouet (1964); some of the preparations were further subfractionated into soluble and insoluble fractions after being subjected to dialysis (overnight at $0^{o}C$) against 1 mM $NaHCO_3$ solution and to centrifugation at 226,000 x g

Table 1

Phospholipase Activities in Composite Fractions of Mitochondria and Lysosomes "M+L", Purified Mitochondria "M", and Purified Lysosomes "L".

Fractions	Substrate hydrolyzed	
	%	nano moles
M+L	35.10 ± 4.80	6.70 ± 0.40
M	11.70 ± 4.40	2.30 ± 0.90
L	51.50 ± 7.40	9.40 ± 1.30

The enzymes were determined in the presence of 8 mM $CaCl_2$, at pH 8.0, with 100 µg or protein and the incubation time was 30 min. Results with "M+L" were obtained from seven separate preparations, that of "M" with eleven separate preparations, and "L" with four separate preparations. The numbers represent the arithmetic mean and their corresponding standard deviations.

for 30 minutes. The lysosomal enzymes were purified 35-53 fold in lysosomes prepared by the floatation method. Assays of cytochrome oxidase, glucose-6-phosphatase and urate oxidase showed that their activities never exceeded 0.9% of the homogenate activities.

Results

The compiled results on phospholipase activities obtained from several separate preparations of "M+L" fractions, purified mitochondria and purified lysosomes are presented in Table 1. The phospholipase found in purified mitochondria (pH 8.0, with $CaCl_2$) was low, relative to its activities found either in "M+L" fractions or in purified lysosomes.

Figure 1 presents two pH curves of phospholipase activities in purified

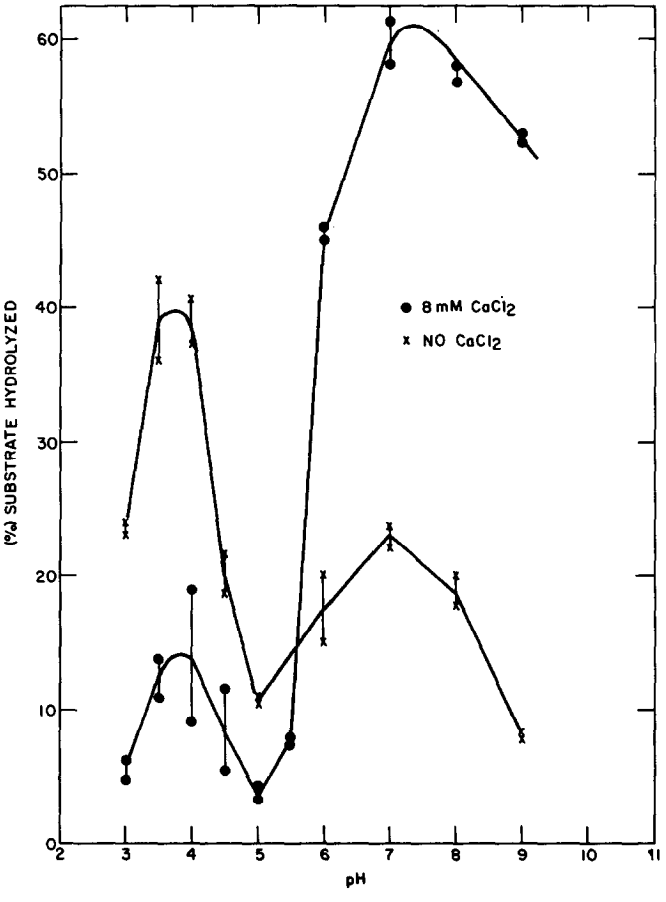

Fig. 1 pH curves of phospholipases in lysosomes. ●----, phospholipases determined in the presence of 8 mM $CaCl_2$, with 18.3 nano moles of substrate. x----, phospholipases determined in the absence of $CaCl_2$, with 25.4 nano moles of substrate. The duplicates are separately expressed at each point.

lysosomes. One was determined in the presence of 8mM $CaCl_2$, and the other in its absence. In the presence of $CaCl_2$, very low enzymatic activities were obtained between pH 3.5 and 4.0; a sharp increase in activity was obtained beyond pH 5.0, with a maximum between the pH values of 7.0 and 8.0. In the absence of $CaCl_2$, however, a sharp peak was found at pH 3.5-4.0; whereas, the activities obtained in the alkaline pH range were decreased to about 50% of that found in the presence of $CaCl_2$.

Figure 2 presents pH curves of phospholipase activities in the soluble as well as in the insoluble fractions obtained from purified lysosomes.

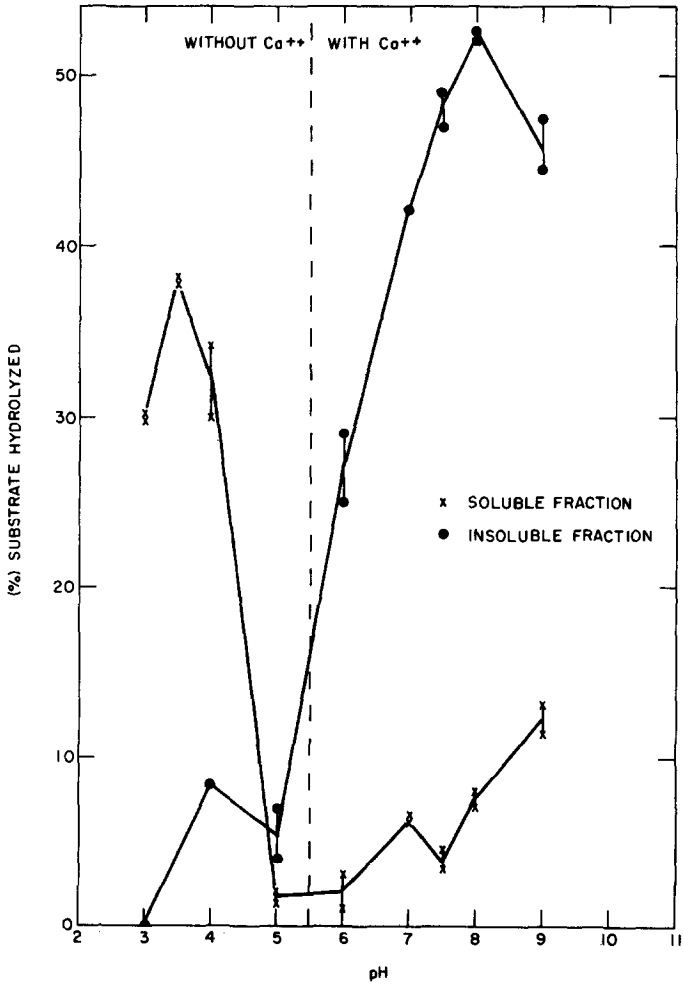

Fig. 2 pH curves of phospholipases in the sub-fractions of lysosomes. •----, phospholipases in the insoluble fraction; x----, phospholipases in the soluble fraction. The enzymes determined between pH 3.0 and pH 5.0 were done in the absence of $CaCl_2$, and that from pH 6.0 to pH 11.0 were done in the presence of 8 mM $CaCl_2$.

Acid phospholipase is demonstrated in the soluble fraction whereas the "alkaline" phospholipase is located in the insoluble fraction.

The percentages of hydrolysis products, i.e., LPE and FFA, obtained from the phospholipases of lysosomes are presented in Figure 3. At pH 4.0, the hydrolysis products are: ∿30% labeled LPE and ∿70% labeled FFA; whereas at pH 7.0 and 8.0 there were no labeled lysocompounds. These results indicate that the phospholipase A with a pH optimum at 4.0 has A_1 and A_2 activities, while the enzyme with a pH optimum between 7.0 and 8.0 has only A_2 activities.

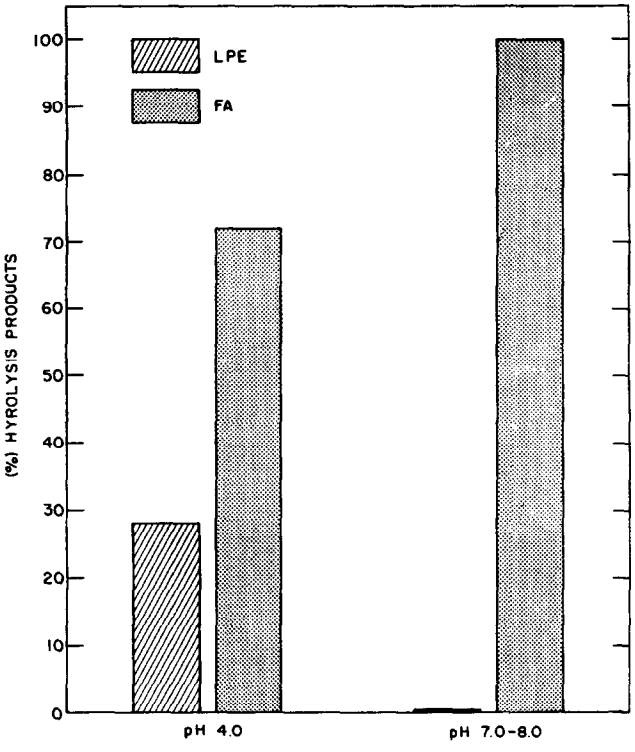

Fig. 3 Hydrolysis products obtained by phospholipases of lysosomes. The enzymes were determined with 100 µg of protein, 20 nano moles of substrate and the incubation time was 30 min. ▨----, lyso-phosphatidyl ethanolamine; ▩----, free fatty acids.

Discussion

A membrane-bound phospholipase A with specific activity for the fatty acid at the 2-position is indicated by these results. That Mellors and Tappel (1967) found very low activities in their rat liver lysosomal membranes seems understandable because in their enzymatic determinations of the lysosomal membranes, only acidic buffers were used, and no $CaCl_2$

was present. For the same reason, this alkaline phospholipase A was not detected by Stoffel and Trabert (1969) when they assayed with unfractionated lysosomes.

Our results (figure 3) confirmed the findings of Mellors and Tappel (1967) as well as those of Stoffel and Trabert (1969); namely, that the acid phospholipase (s) A in liver lysosomes are capable of hydrolyzing both of the fatty acids (1 and 2-position); even though the use of a substrate labeled only at the 2-position is not ideal for activities hydrolyzing the fatty acid of the 1-position. This acid phospholipase was found to be inhibited by Ca^{++} (Fig. 1) which is in accordance with the results of Waite *et al*, (1969).

It is clear from our data that mitochondria have low phospholipase A activities in comparison with those of lysosomes. Whether the phospholipase A found in mitochondria was from contaminations of lysosomal membranes cannot be ascertained at present. The lack of a suitable marker enzyme, unique to lysosomal membranes, rendered solution of this problem difficult. Work in progress may clarify this point.

A phospholipase with an optimal activity close to physiological pH (obtained under our experimental conditions), activated by Ca^{++}, and specifically localized in the lysosomal membranes is an extremely interesting finding. One could postulate that this enzyme, mediated by Ca^{++}, might play a role in the initial binding of the lysosomes to membranes of other subcellular particles, as it is known that mitochondrial and microsomal membranes contain high percentage of phospholipids (Rouser *et al*, 1968). This binding would enable the subsequent fusion between lysosomes and other particles. This fusion, in turn, would allow the ultimate release of the soluble acid hydrolases from the lysosomes.

ACKNOWLEDGMENTS

Much of the senior author's contribution to this study was accomplished during her leave of absence from Argonne National Laboratory. She thanks Prof. L. L. M. van Deenen of the Laboratory of Biochemistry, State University of Utrecht, for his kind hospitality and helpful advice. Thanks are also due to Prof. C. de Duve for extending his laboratory facilities at Louvain University for the preparations of lysosomes. The generous help offered by Dr. D. Thinès- Sempoux and Prof. H. Beaufay, also of Louvain University, during the preparations of lysosomes, is gratefully acknowledged.

The authors thank Mr. A. J. Aarsman of the Laboratory of Biochemistry, State University of Utrecht, for his valuable technical assistance in some experiments.

This work was supported in part by the U. S. Atomic Energy Commission.

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