Phospholipase A Activity of Lysosomes of Rat Myocardial Tissue*

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ABSTRACT: Lysosomes were isolated by isopycnic sucrose gradient centrifugation from myocardial tissue of control rats and rats injected with Triton WR-1339. As measured by the shifts in distribution of acid phosphatase, β-glucuronidase, and one of the phospholipases A, three populations of lysosomes were found which had engorged Triton. A less dense population contained relatively more β-glucuronidase, whereas a denser population contained relatively more acid phosphatase. The majority of the lysosomes were unaffected by Triton. Based on the observations made on rat skeletal muscle (Canonico, P. G., and Bird, J. W. C. (1970), *Cytobios 14*, 23), it is suggested that four groups of lysosomes of different cellular origin exist in rat myocardial tissues, and that more than one type of lysosome may be derived from the same cell, probably the macrophage. The lysosomal phospholipases A are optimally active at pH 4.0 with 2.0 mM EDTA and at pH 5.0 with 2.0 mM Ca^{2+}. These characteristics are unlike those reported earlier in rat heart (Weglicki *et al.* (1971), *Biochem. Biophys. Acta 231*, 512) but closely resemble the phospholipase A of rat liver lysosomes (Franson *et al.* (1971), *Biochemistry 10*, 1942) and of alveolar macrophage from rabbit. Since some of the lysosomes which contain the phospholipases shifted in distribution with Triton treatment as did those particules which contain both the acid phosphatase and β-glucuronidase, it is suggested that: (1) these phospholipases A are in part of macrophage lysosomal origin, and (2) that the phospholipases A are not distributed to an equal extent between the lysosomal populations.

Recently, Weglicki *et al.* (1971) described the localization of phospholipases A_1 and A_2 in microsomes and mitochondria from rat heart. No studies were reported on the phospholipase A active at acid pH values from myocardial tissue at that time, however. Phospholipases A_1 and A_2 active at pH 4.0 have been detected in rat liver lysosomes (Stoffel and Trabert, 1969; Franson *et al.*, 1971). It therefore became of interest to see if phospholipases similar to those found in rat liver lysosomes exist in the lysosomes of myocardial tissue.

Lysosomes of muscle cells and macrophages from skeletal muscle have been separated by the selective uptake of Triton WR-1339 by macrophage lysosomes (Pollack and Bird, 1968; Canonico and Bird, 1970). Skeletal muscle cell lysosomes, on the other hand, do not phagocytize Triton. Also, within one cell type, the rabbit heterophil leukocyte, at least three groups of lysosome-like granules with different enzymatic compositions have been found (Baggiolini *et al.*, 1969). In addition, two morphologically distinct populations of rat liver lysosomes have been isolated by Franson *et al.* (1971). These results demonstrate the heterogenity of lysosomal characteristics and origin and, therefore, the necessity of careful characterization of lysosomes when cellular localization of an enzyme is being determined.

In this work, lysosomes from rat myocardial tissue are studied by isopycnic sucrose gradient centrifugation. Distribution of lysosomes which contain β-glucuronidase (EC 3.2.1.3) and acid phosphatase (EC 3.1.3.2) from control rats and rats treated with Triton WR-1339 indicate the presence of possibly four populations of lysosomes, presumably of both muscle cell and macrophage origin. In these preparations which contain lysosomes, phospholipases A_1 and A_2 are found with optimal activity in the acid pH range. The similar shift in the distribution of the phospholipases A and the lysosomal marker enzymes due to phagocytosis of Triton as well as their acid pH optima indicate that the phospholipases A are of lysosomal origin and suggest that these enzymes may be associated primarily with macrophage lysosomes. Indeed, phospholipases with similar characteristics were found in alveolar macrophages.

Materials and Methods

Preparation of Lysosomes. Six male Wistar rats (150–200 g) were injected with 225 mg of Triton WR-1339 3.5 days prior to sacrifice. Six rats injected with an equal volume of isotonic saline were controls. The rats were killed and the hearts were rapidly removed, allowed to contract in sucrose to pump out the blood, minced, and homogenized with a Ten-Broeck homogenizer in a total volume of 50 ml of 0.25 M sucrose containing 3 mM $MgCl_2$ and 20 mM Tris-HCl (pH 7.2). All steps were performed at 0–4°. The homogenate was filtered through two layers of cheesecloth and the filtrate was centrifuged at 800*g* for 10 min. The supernatant fluid was decanted and was centrifuged at 10,000*g* for 20 min. This yielded a lysosome-rich pellet and a supernatant fraction. Six milliliters of the pellet which had been resuspended in 24 ml of 0.25 M sucrose was layered over 26 ml of a continuous gradient from 0.73 to 1.46 M sucrose. Three milliliters of 1.93 M sucrose was used as a cushion at the bottom of the tube. The samples were centrifuged at 27,000 rpm (100,000*g*) for 4.0 hr in a Beckman SW-27 swinging-bucket rotor. Six fractions (6.5 ml/fraction) were collected from the top of the centrifuge tube by puncturing the bottom of the tube and pumping 1.93 M sucrose from below. The lysosomes were ruptured by freezing, sonication, or dialysis for 12 hr against 6 l. of 1 mM EDTA (pH 7.2). Acid phosphatase and β-glucuronidase were assayed with fractions which were frozen and thawed three times. Phospholipases A were active only in preparations which had been osmotically ruptured.

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TABLE I: Protein and Lysosomal Marker Enzymes of Tissue Fractionation.[a]

Fraction	Protein		Acid Phosphatase		β-Glucuronidase	
	Total (mg)	%	TA	%	TA	%
Control						
Filtered homogenate	556	100	2459	100	6164	100
Lysosome-rich pellet (10,000*g*)	104	19	446	18	804	13
Supernatant (10,000*g*)	173	31	1492	61	3840	62
Triton						
Filtered homogenate	618	100	2628	100	7990	100
Lysosome-rich pellet (10,000*g*)	117	19	579	22	682	9
Supernatant (10,000*g*)	191	31	1528	58	5120	64

[a] Protein and marker enzymes were assayed by methods described in Materials and Methods. Total activity (TA) is expressed as nanomoles of product formed per minute for acid phosphatase and nanomoles of product formed per hour for β-glucuronidase. Per cent of activity is calculated as the total activity of a given fraction divided by the total activity in the filtered homogenate times 100. These data are the average of seven experiments.

Alveolar macrophages were isolated from a normal New Zealand white rabbit (2.0 kg) (Myrvik *et al.*, 1961). On microscopic examination the cell suspension was 95% alveolar macrophage. Packed cells (0.1 ml) were resuspended in 3.5 ml of isotonic saline.

Enzyme Assays. Phospholipase A was assayed as described by Waite and van Deenen (1967). Phospholipases A_1 and A_2 specifically hydrolyze the fatty acid ester linkages in the C-1 and C-2 positions of phospholipids, respectively, to form their monoacyl analogs. Reaction mixtures contained in a total volume of 1.5 ml (unless otherwise specified) 2 nmoles of 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine (10,000 cpm added as an ultrasonic suspension), 2 μmoles of EDTA or 2 μmoles $CaCl_2$, 100 μmoles of sodium acetate, and either 0.20 or 1.0 ml of the enzyme. The reaction mixtures were incubated for 20 min at 37° and the lipids were extracted and separated by thin-layer chromatography on silica gel G plates. The chromatograms were first developed in chloroform–petroleum ether (bp 30–60°)–acetic acid (70:30:2, v/v) and then in chloroform–methanol–H_2O (70:30:4, v/v) (Franson *et al.*, 1971). Chromatograms were then stained with I_2 vapor and the silicic acid that contained the radioactive compounds was placed in scintillation vials containing Omnifluor scintillation mixture and a thixotropic gel.

The specificity of macrophage phospholipases A_1 and A_2 was determined by the method of Waite and van Deenen (1967) using a mixture of two labeled substrates. Incubation mixtures contained in a total volume of 1.0 ml, 100 μmoles of sodium acetate buffer (pH 4.0), 2 μmoles of EDTA, 50–100 μg of protein of homogenized alveolar macrophages, and a total of 25 nmoles of 1-[^{3}H]palmitoyl-2-acyl-3-glycerophosphorylethanolamine (1×10^5 dpm) and 1-acyl-2-[^{14}C]-linoleoyl-3-glycerophosphorylethanolamine (6×10^3 dpm). Radioactive lipids were separated by thin-layer chromatography on silica gel G plates and the radioactive compounds were extracted from the silicic acid by the method of Waite and van Deenen (1967). Hydrolysis of the 1-[^{3}H]palmitoyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine by snake venom (phospholipase A_2) (*Crotaleus adamanteus*) demonstrated that 97% of the ^{14}C was in the C-2 position of the 1,2-diacyl-3-glycerophosphorylethanolamine and 77% ^{3}H was in the C-1 position.

Acid phosphatase (EC 3.1.3.2 lysosomal marker) was determined by the method of Gianetto and deDuve (1955). Inorganic phosphate was measured by the turbidimetric procedure of Eibl and Lands (1969). Rotenone-insensitive NADH–cytochrome *c* reductase (EC 1.6.99.3 microsomal and mitochondrial marker) and cytochrome oxidase (EC 1.9.3.1, mitochondrial marker) were assayed spectrophotometrically by the method of Sottocasa *et al.* (1967). β-Glucuronidase (EC 3.2.1.3, lysosomal marker) activity was determined by a modification of the method of Talalay *et al.* (1946) as described by Canonico and Bird (1969). Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Materials. We synthesized 1-[^{3}H]palmitoyl-2-acyl-3-glycerophosphorylethanolamine and 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine by the procedure of Waite and van Deenen (1967). Triton WR-1339 was purchased from Ruger Chemical Co., New York.

Results

Table I compares the recovery of protein, acid phosphatase, and β-glucuronidase in the filtered homogenate, and the lysosome-rich pellet and supernatant. Since most of the marker enzymes were found in the soluble fraction, it appears as if the majority of the lysosomes were disrupted by homogenization. The total activity of both lysosomal enzymes was higher in the Triton-treated homogenates than in the control; β-glucuronidase increased 23%, whereas acid phosphatase increased 6%. Triton treatment did not appreciably influence the distribution of the enzymes between the supernate and pellet.

The particulate fraction was sonicated or frozen and thawed three times to demonstrate the latency of the lysosomal enzymes in this fraction. About 50% of the acid phosphatase and 90% of the β-glucuronidase in the lysosome-rich fraction were solubilized by these treatments whereas most of these enzymes remained in the particles of an untreated lysosome-rich fraction. This demonstrates that most of the lysosomes in the 10,000*g* pellet were intact.

The pH for optimal phospholipase A activity of the lysosome-rich pellet with Ca^{2+} or EDTA is shown in Figure 1. Two phospholipases, A_1 and A_2, have optimal activity at pH 4.0 in the presence of 2.0 mM EDTA. The phospholipase A_2

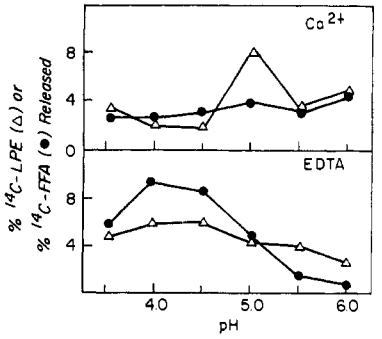

FIGURE 1: Optimal pH values for rat heart phospholipase A activity of resuspended postnuclear pellet (10,000*g*). Incubation mixtures contained 1.2 mg of resuspended postnuclear pellet (10,000*g*), 2 nmoles of 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine (10,000 cpm), 100 μmoles of sodium acetate buffer, and 2 μmoles of Ca^{2+} or EDTA in a total volume of 1.0 ml. Reaction mixtures were incubated at 37° for 20 min. [^{14}C]LPE and [^{14}C]FFA refer to 2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine and [^{14}C]linoleic acid, respectively. All values presented have been corrected for nonenzymatic hydrolysis of substrate.

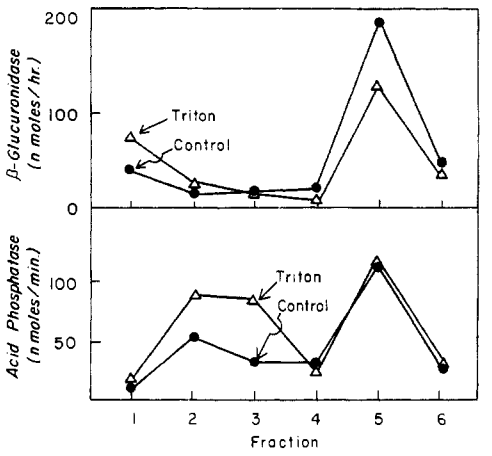

FIGURE 2: Distribution patterns of β-glucuronidase and acid phosphatase after isopycnic sucrose gradient centrifugation. Resuspended postnuclear pellet (6.0 ml; 10,000*g*) was layered above a continuous sucrose gradient (25–50% w/v sucrose). Isolation of the fractions and the enzymatic assay procedures were described in Materials and Methods. Total activities are expressed on the ordinate as nanomoles of product formed per minute for acid phosphatase and nanomoles of product formed per hour for β-glucuronidase.

is more active than phospholipase A_1 and has optimal activity over a narrower pH range. In the presence of 2.0 mM Ca^{2+}, a phospholipase A_1 is observed with optimal activity at pH 5.0. The activity at the 2 position is one-half that at the 1 position and could be due to a lysophospholipase. The phospholipases A active at pH 4.0 are inhibited 50–75% by 2.0 mM Ca^{2+}.

The recovery of phospholipase A in the lysosome-rich and supernatant fractions is shown in Table II. Most of the phospholipases were found in the soluble fraction, as were the lysosomal marker enzymes. Triton treatment increased the apparent amount of the phospholipases active at pH 4.0 but not that active at pH 5.0. The decrease in activity at pH 5.0 could be due to inhibition of that enzyme by Triton.

Figures 2 and 3 show the distribution of acid phosphatase, β-glucuronidase, cytochrome oxidase, and rotenone-insensitive NADH–cytochrome *c* reductase in control and Triton treated preparations after isopycnic sucrose gradient centrifugation. Fraction 5 of the control contains the majority of both lysosomal activities. Triton treatment causes a decrease in the density of some of the lysosomes which contain β-glucuronidase and acid phosphatase. This indicates that these enzymes are in macrophage lysosomes. β-Glucuronidase increased primarily in fraction 1, whereas acid phosphatase increased in fractions 2 and 3, which suggests that the acid phosphatase containing lysosomes have taken up less Triton, relative to that taken up by the β-glucuronidase-rich lysosome. Since muscle cell lysosomes have no known phagocytic function, those lysosomes whose densities did not shift (fraction 5)

TABLE II: Phospholipase A Activity of the 10,000*g* Supernatant and Particulate Fraction.[a]

Fraction	Phospholipase A Total Activity		
	pH 4.0		pH 5.0
	A_1	A_2	A_1
Control			
Lysosome-rich supernatant (10,000*g*)	186	1114	1674
Pellet (10,000*g*)	15	45	291
Triton			
Lysosome-rich supernatant (10,000*g*)	437	1242	1207
Pellet (10,000*g*)	50	195	111

[a] Phospholipase A was determined as described in Materials and Methods. Incubation mixtures contained 1.0 ml of supernatant fluid or 0.2 ml of resuspended pellet; assays at pH 4.0 were performed in the presence of 2.0 mM EDTA and at pH 5.0 in 2.0 mM Ca^{2+}. Total activity is expressed as nanomoles of ^{14}C product formed per hour.

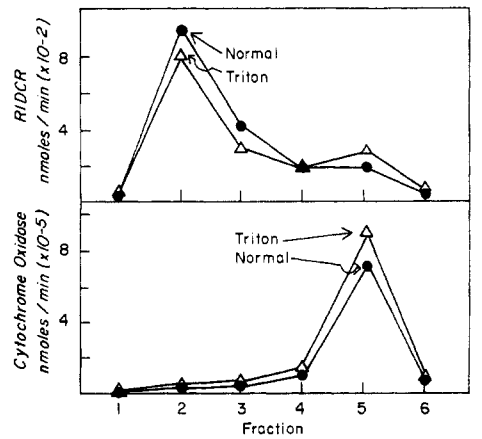

FIGURE 3: Distribution patterns of cytochrome oxidase and rotenone insensitive NADH–cytochrome *c* reductase (RIDCR) after isopycnic sucrose gradient centrifugation. Resuspended postnuclear pellet (6.0 ml., 10,000*g*) was layered above a continuous sucrose gradient (25–50% w/v sucrose). Isolation of the fractions and the enzymatic assay procedures were described in Materials and Methods. Total activities are expressed on the ordinate as nmoles of reduced cytochrome *c* oxidized/min $\times 10^{-5}$ for cytochrome oxidase and nmoles of oxidized cytochrome *c* reduced/min $\times 10^{-2}$ for RIDCR.

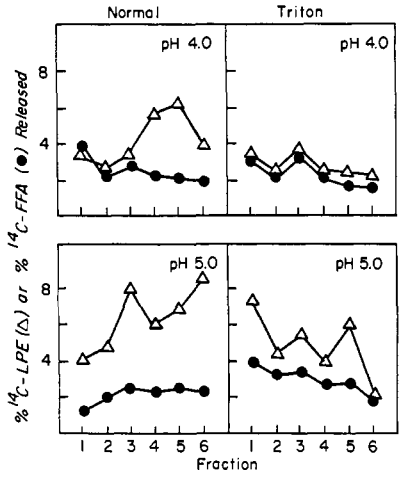

FIGURE 4: Distribution patterns of phospholipases A after sucrose gradient centrifugation. Resuspended postnuclear pellet (6.0 ml; 10,000*g*) was layered above a continuous sucrose gradient (25–50% w/v sucrose). Isolation of the fractions was described in Materials and Methods. Incubation mixtures contained 1.0 ml of each fraction from the gradient, 2 nmoles of 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine (10,000 cpm), 100 μmoles of sodium acetate buffer, and 2 μmoles of Ca^{2+} at pH 5.0, or 2 μmoles of EDTA at pH 4.0 in a total volume of 1.5 ml. Reaction mixtures were incubated at 37° for 20 min. [^{14}C]LPE and [^{1} C]FFA refer to 2-[^{14}C]linoleoy-3-glycerophosphorylethanolamine and [^{14}C]linoleic acid, respectively. All values presented have been corrected for nonenzymatic hydrolysis of substrate.

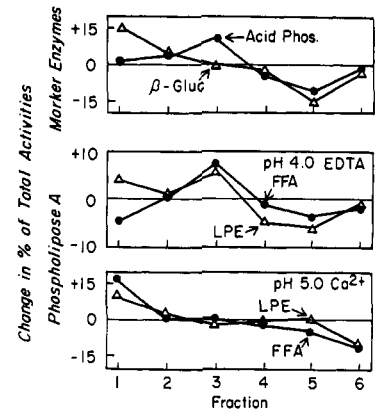

FIGURE 5: Changes in the sucrose gradient distribution pattern of lysosomal marker enzymes and phospholipase A as a result of Triton treatment. Values were obtained from Figures 2 and 4. Per cent of activity for each enzyme was calculated for the control and the Triton-treated preparation, as the total activity of each fraction divided by the total activity of the entire gradient. The change in per cent of total activity (ordinate) is calculated by subtracting the per cent of activity in each fraction of the control from the per cent of activity on the corresponding fraction of the Triton-treated preparation.

are presumed to be of muscle origin. The total activity of marker enzymes which went onto the sucrose gradients was not identical, so these data reflect qualitative changes in distribution only. The extent of these changes in distribution varied considerably between experiments which could be the results of a difference in the uptake of Triton. Generally, more of the β-glucuronidase shifted than was found in the experiment reported here. Triton had no effect on the densities of the mitochondria or microsomes (Figure 3).

Figure 4 shows the distribution of the phospholipases A after gradient centrifugation. At pH 4.0 the phospholipase A_1 activity of fractions 4 and 5 in the control is decreased after Triton treatment with a relative increase in the lighter fractions. At pH 5.0, the recovery of phospholipase A_1 increased in fraction 1 with a major decrease in fraction 6. A significant amount of activity remained in fraction 5. The presence of the phospholipase A_1 active at pH 5.0 in fraction 6 remains an anomaly since little of the marker enzymes was found in this fraction. It does appear, however, that this enzyme, found in both fractions 5 and 6, is associated with both the macrophage and muscle cell lysosomes since Triton treatment does cause a dramatic shift in the activity from fraction 6 to 1, but no concomitant shift of activity from fraction 5.

Since the total activities in the preparations from control and treated rats were different, the relative distributions of the enzymes due to Triton treatment were compared as the per cent of the total recovered activity found in each fraction (Figure 5). Lysosomes which contain phospholipase A active at pH 5.0 increased in fraction 1 as the result of Triton treatment as did those containing β-glucuronidase. However, the shift in activity is from fraction 6 rather than from fraction 5 as with the β-glucuronidase. At pH 4.0 the shift in distribution of phospholipase A_1 resembles that of both β-glucuronidase and acid phosphatase which suggests that the phospholipase A_1 is located in two different populations of macrophage lysosomes. The shift of phospholipase A_1, on the other hand, is more similar to that of acid phosphatase.

Since these data suggest the lysosomal phospholipases A are associated primarily with macrophages, the pH for optimal activity of phospholipase A and the effect of Ca^{2+} on normal rabbit alveolar macrophages were determined (Figure 6). These enzymes have characteristics which are remarkably similar to those found in heart (Figure 1) with regard to pH optima, Ca^{2+} requirement, and positional specificity. Two phospholipases, A_1 and A_2, have optimal activity at pH 4.0 in the presence of 2.0 mM EDTA. With 2.0 mM Ca^{2+}, a phospholipase A_1 with optimal activity at pH 5.0 is seen. Some hydrolysis of the substrate also occurred at the two position. On the basis of specific activities (nanomoles of

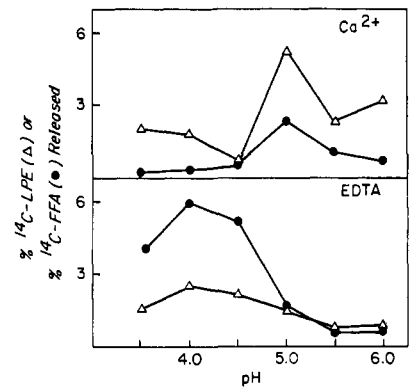

FIGURE 6: Optimal pH values for phospholipase A activity of rabbit alveolar macrophage. Cells were isolated by a method described in Materials and Methods.Incubation mixtures contained 50 μg of macrophage lysate (frozen and thawed eight times), 50 nmoles of 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine (10,000 cpm), 100 μmoles of sodium acetate buffer, and 2 μmoles of Ca^{2+} or 2 μmoles of EDTA in a total volume of 1.0 ml. Reaction mixtures were incubated at 37° for 20 min. [^{14}C]LPE and [^{14}C]FFA refer to 2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine and [^{14}C]linoleic acid, respectively. All values presented here have been corrected for nonenzymatic hydrolysis of substrate.

TABLE III: Hydrolysis of 1-[^{3}H]Palmitoyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine by Alveolar Macrophage Homogenates.[a]

Products	Calcd (%)	Found (%) ^{14}C	Found (%) ^{3}H
Free fatty acid		30.9	35.6
Monoacylglycerophosphorylethanolamine		5.8	3.7
Glycerophosphorylethanolamine	28.5		

[a] Phospholipase A was determined as described in Materials and Methods. Per cent hydrolysis is calculated as the disintegrations per minute recovered as free fatty acid and monoacyl-3-glycerophosphorylethanolamine divided by the total radioactivity recovered times 100. Lysophospholipase activity was calculated as the difference between monoacyl-3-glycerophosphorylethanolamine and free fatty acid divided by 2 so that the amount of the original phosphatidylethanolamine is 100%. These data are the average of four experiments and have been corrected for nonenzymatic hydrolysis.

substrate hydrolyzed per minute per milligram of protein), the phospholipases A of alveolar macrophage are 400–500 times more active than the myocardial preparations.

Hydrolysis by macrophage homogenates of phosphatidylethanolamine with [^{3}H]palmitic acid in the 1 position and [^{14}C]linoleic acid in the 2 position yielded both [^{3}H]- and [^{14}C]monoacylglycerophosphorylethanolamines which confirms the presence of both phospholipase A_1 and A_2 (Table III). However, about 80% of both monoacylglycerophosphorylethanolamines were further hydrolyzed which is indicative of lysophospholipase activity. It is not possible to determine whether the hydrolysis of the monoacylglycerophosphorylethanolamine is due to a lysophospholipase or a phospholipase A with lysophospholipase activity. Generally, more monoacylglycerophosphorylethanolamine was found with incubations using 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine (*cf.* Figure 6); we attribute this difference to the lower substrate concentrations used in the mixed-labeled ^{3}H, ^{14}C incubations.

Discussion

Using the approach of Canonico and Bird (1970), we have separated muscle cell and macrophage lysosomes from myocardial tissue. Furthermore, we have separated two, and possibly three, groups of lysosomes from the macrophage with different enzymatic compositions. Phospholipase A_1 active at pH 4.0 is increased in both populations of lysosomes whose densities shift due to Triton treatment (fractions 1 and 3), whereas phospholipase A_2 active at pH 4.0 is found in the β-glucuronidase-rich lysosome (fraction 1). Similar enzymes were found in rat liver lysosomes (Franson *et al.*, 1971), and we presume that these lysosomes are from the Kupffer cell which is of macrophage origin (Boak *et al.*, 1968). It is of interest that the phospholipase A_1 active at pH 5.0 was not found in rat liver lysosomes (Franson *et al.*, 1971) even though we conclude that some of this enzyme is in the macrophages from both alveolar and heart preparations. However, we conclude that some of the phospholipase A_1 active at pH 5.0 is localized in heart lysosomes which are not affected by Triton treatment (fraction 5).

The conclusion that the phospholipases reported here (with the exception of some of the enzyme active at pH 5.0) are from the macrophage is supported by the finding that alveolar macrophages, which are rich in lysosomes, have high phospholipase A_1 and A_2 activity under the same conditions. Similar results were found by Elsbach (1966) using ^{32}P-labeled lecithin, even though he was not able to demonstrate the positional specificity of hydrolysis. The 400- to 500-fold difference between the specific activities of alveolar macrophage and heart suggests that a small population of lysosomes derived from the macrophage may exist and account for some phospholipase activities observed in the heart.

Phospholipase A_1 and A_2 activities were reported in microsomes and mitochondria of rat heart (Weglicki *et al.*, 1971). Both enzymes have optimal activity at higher pH values (mitochondria, A_2, pH 9.5, microsomes, A_1 and A_2, pH 7.5, and lesser activity at pH 5.5–6.0) and require Ca^{2+} for optimal activity. In our lysosomal preparations the phospholipases A active at pH 4.0 required EDTA for optimal activity and were markedly inhibited at 2.0 mM Ca^{2+}. Phospholipase A_2 activity at pH 5.0, however, is optimal in the presence of 2.0 mM Ca^{2+}. The Ca^{2+}-dependent phospholipase A of the microsomes (Weglicki *et al.*, 1971) had activity between pH 5.5 and 6.0, in addition to the major activity observed at pH 7.5. It is possible that the acid-active phospholipase A reported earlier in microsomes (Weglicki *et al.*, 1971) is of lysosomal origin.

Acknowledgments

The authors thank Patricia Sisson and Elizabeth Guy for their excellent technical assistance.

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