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PURIFICATION AND SOME PROPERTIES OF RAT SPLEEN PHOSPHOLIPASE A

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SUMMARY

A phospholipid-degrading enzyme, phospholipase A (EC 3.1.1.4), has been purified from rat spleen. This enzyme has the following properties: (1) a specificity for the fatty acid at the 2-position, (2) activation by Ca^{2+}, (3) a pH optimum around 7.0, (4) inhibition by various divalent cations such as Mg^{2+}, Hg^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+}, (5) an isoelectric point around 7.4, (6) a molecular weight of 15 000, (7) inhibition by Triton X-100 and EDTA, (8) sensitivity to inhibition by deoxycholate when phosphatidylcholine is used as substrate, and insensitivity to deoxycholate inhibition when the substrate is phosphatidylethanolamine, (9) increased resistance, in the presence of fat, to heat denaturation at low pH.

INTRODUCTION

Phospholipase A (EC 3.1.1.4) is a phospholipid-degrading enzyme that produces highly hemolytic lysophosphatides from phospholipids[1]. First discovered in snake venom[2], this enzyme also exists in various mammalian tissues, including the spleen[3]. Due to the improvement of the enzyme determination method[4], phospholipases of different properties have been demonstrated not only in eukaryotes but also in prokaryotes such as *Escherichia coli*[5,6]. In mammalian cells as well as in bacteria, phospholipases with alkaline pH optima are generally membrane-bound[5,7–10].

To determine the biological function of spleen phospholipase A in relation to the process of erythrocyte destruction and removal, it is necessary to study this enzyme in its purified form. This paper is a preliminary report on the purification procedure for a spleen phospholipase A; a brief characterization of this enzyme is also presented.

MATERIALS AND METHODS

[U-^{14}C]Phosphatidylethanolamine and [U-^{14}C]phosphatidylcholine with specific activities $>$ 2 Ci/mole, were obtained from Applied Science Laboratories, Inc. (State

College, Pa.). They were further purified by thin-layer chromatography[8]. The former was used as the routine substrate for phospholipase A determinations, but the latter was also used in a few experiments. 1-Palmitoyl-[2-^{14}C]linoleoylglyceryl-3-phosphorylethanolamine synthesized according to Van Den Bosch *et al.*[4] was used to determine the enzyme specificity. Purified snake venom *(Vipera russelli)* phospholipase A_2 was obtained from Sigma Chem. Co. (St. Louis, Mo.). [U-^{14}C]1-acylglyceryl-phosphorylethanolamine was isolated by thin-layer chromatography from the degradation products obtained by incubating the venom phospholipase A with [^{14}C]phosphatidylethanolamine as substrate. [^{14}C]1-Acylglycerylphosphorylethanolamine was used to test the existence of lysophospholipase activity. Sephadex gels used for column chromatography were from Pharmacia, Inc. (Piscataway, N.J.). Ampholine solutions used for the isoelectrofocusing systems were obtained from LKB Instruments, Inc. (Rockville, Md.). An LKB-8300 A Uvicord II was used to monitor the absorbance of the column eluents at 280 nm.

COBS^{R}CD^{R}(SD) strain rats obtained from the Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) were used. Spleens weighing between 80–150 g from about 100 rats of both sexes with some surrounding fat still attached, were used for each purification experiment.

The procedure for enzyme incubation and the method for subsequent lipid separation by thin-layer chromatography were the same as previously described[8]. Protein content was determined by the method of Lowry *et al.*[11].

The purification procedure is as follows: The rat spleens were rapidly homogenized in 4 vol. of ice-cold distilled water in a Polytron type PT 20 homogenizer. The homogenate (H) was sonicated several times in an ice bath with a Branson Sonifier Model W 185, each time for a short duration of 2–3 min to avoid excessive heating. The sonicated homogenate was then centrifuged in a Sorvall RC2B centrifuge at 10 000 rev./min for 10 min; the pellet obtained was discarded and the supernatant (S_1) was dialyzed against twice-distilled water at 0 °C overnight. The dialyzed supernatant (S_2) was acidified to pH 5.0 with 1 M HCl and heated to 65 °C for 5 min. The mixture was rapidly cooled to 0 °C and centrifuged at 20 000 rev./min (45 000 × *g*) in a No. 30 rotor for 30 min in a Spinco centrifuge Model L2-50. The clear supernatant (S_3) was chromatographed on Sephadex G-50 in a 5 cm × 90 cm

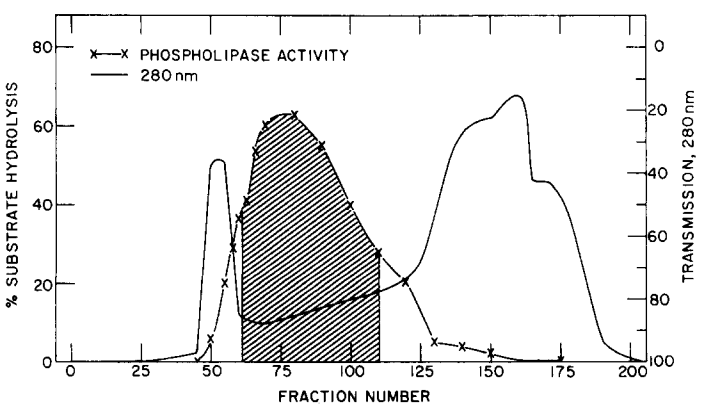

Fig. 1. Elution pattern of phospholipase A from a Sephadex G-50 column chromatography with water as eluent. ×—×, Enzyme activity; ———, absorbance at 280 nm.

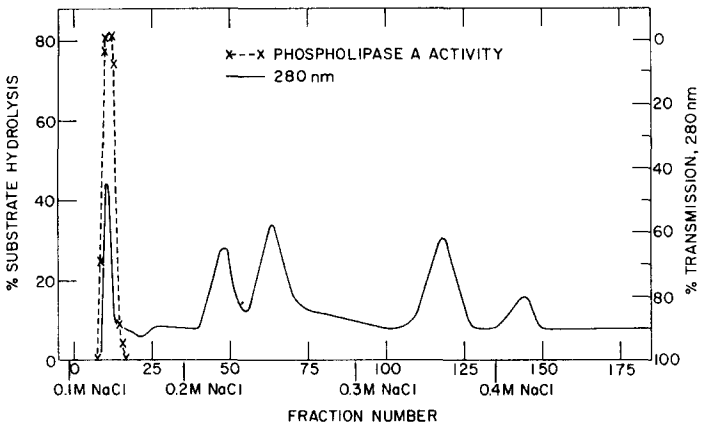

Fig. 2. Elution pattern of phopsholipase A from a DEAE-Sephadex A-25 column chromatography at pH 8.5. ×---×, Enzyme activity; ———, absorbance at 280 nm.

ascending column at 4 °C, with distilled water as eluent; two major protein peaks were obtained (Fig. 1), and the highest phospholipase activities were found in fractions between the two major protein peaks; these fractions were pooled for further chromatography in a DEAE-Sephadex A-25 column. For the DEAE-Sephadex column, 0.1 M Tris–HCl buffer (pH 8.5) was used as eluent. After the appearance of a small breakthrough peak, the column was eluted stepwise by 0.1–0.5 M NaCl. One major sharp peak of protein was eluted with 0.1 M NaCl, and the phospholipase was found to be in the fractions comprising this protein peak (Fig. 2).

The fractions obtained from the DEAE-Sephadex column that contained phospholipase were further purified in a Sephadex G-75 column eluted with 0.1 M

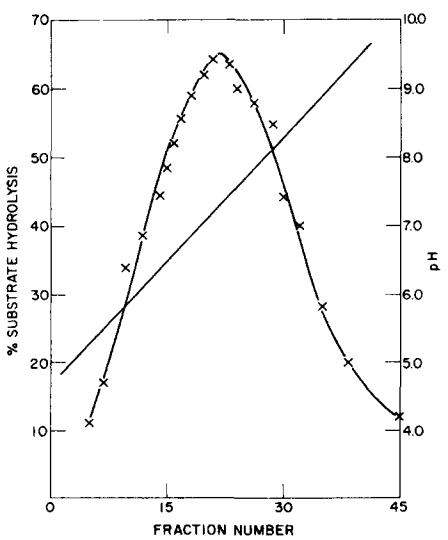

Fig. 3. Electrofocusing of spleen phospholipase A in the pH range of 5 to 9. Enzyme obtained from Sephadex G-75 column chromatography was applied and focused for 72 h at 200 V and 6 °C; then collected in fractions of 2.0 ml. ———, pH gradient; ×—×, enzyme activity.

Tris–HCl buffer (pH 7.0), in 0.1 M NaCl, which yielded a main protein peak containing phospholipase A activity and two to three minor protein peaks.

Fractions obtained from the Sephadex G-75 column containing phospholipase A activity were then pooled, dialysed against distilled water, lyophilized, and subjected to isoelectric focusing. Various pH gradients ranging between pH 3 and pH 10 were used for this system[12]; and a single peak of phospholipase A activity was usually observed (Fig. 3).

Fractions from the electrofocusing column containing phospholipase A activity were applied to a Sephadex G-25 column with 0.1 M KCl, 0.01 M Tris–HCl buffer (pH 7.0), and 10^{-3} M Cleland's reagent as eluent. This step was necessary for removing the ampholine solution in the samples before they were subjected to polyacrylamide gel electrophoresis.

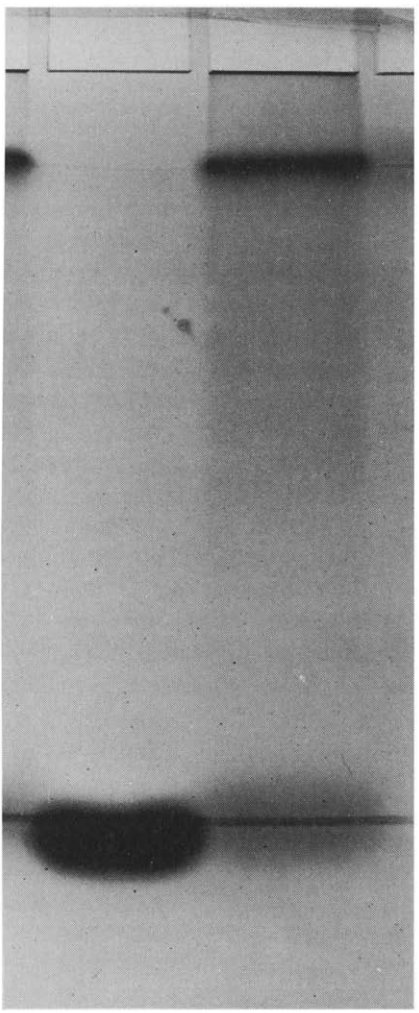

Fig. 4. Polyacrylamide gel electrophoresis of purified spleen phospholipase A. The electrophoresis was performed in Tris–boric acid buffer at pH 9.2, 300 V, 140 mA at 0 °C for 3 h. Left column: a yellow flavoprotein used as front marker. Right column: phospholipase A.

Polyacrylamide gel electrophoresis was performed in buffer (Tris 80 mM, disodium EDTA 2.7 mM, boric acid 6.0 mM) at pH 9.2 on a 5% polyacrylamide gel slab of 18 cm in length, and 3 mm in thickness. The electrophoresis was run at 300 V and 140 mA for approximately 3 h. The gel slab was then removed and fixed in 7% acetic acid for 30 min prior to staining with amido black; the gel was subsequently de-stained by extensive washing first in 7% acetic acid followed by running tap water. Usually a single band was observed (Fig. 4).

The molecular weight of the enzyme was estimated by gel filtration on a 2.5 cm × 40 cm column of Sephadex G-75. The protein standards were those in the molecular weight calibration kit supplied by Pharmacia, Inc., and the procedure followed was as described in the calibration kit instruction manual. The column effluent passed through an ultraviolet monitor and into a fraction collector. The elution volume of the enzyme peak was verified by activity measurements on the collected fractions, as well as by detection of the ultraviolet peak.

Table I shows the yield and the specific activity at various stages during purification.

TABLE I

PURIFICATION OF SPLEEN PHOSPHOLIPASE A

Fraction	*Total protein (g)*	*Enzyme Yield (%)*	*Specific activity (moles/mg protein per min)*
H	39	—	—
S_1	34	—	—
S_2	18	—	—
S_3	8	(100)	0.11
Sephadex G-50	2	80	0.35
DEAE Sephadex	0.2	19	0.82
Sephadex G-75	0.02	10	4.30
Isoelectrofocusing	—	—	5.00

RESULTS AND DISCUSSION

Stability of spleen phospholipase A

During the development of our purification procedure, we accidentally found that if the spleens were homogenized in the presence of some of their surrounding fat, the recovery of the phospholipase activity after being heated at 65 °C for 5 min was high; this resistance to heat was further increased if the spleen homogenate was exhaustively dialysed against distilled water prior to the heating (Table II).

In the presence of fat, crude spleen phospholipase A is relatively heat-resistant in an acidic medium (Fig. 5). Heating at 60 °C for 10 min did not produce a loss of enzyme activity when the incubation medium was at pH 5.0; however, at pH 7.0, only 50% of the enzyme activity remained, and at pH 9.0, a complete loss of activity was observed.

Owing to the protective effect of fat tissue against the heat treatment, purification of the spleen phospholipase was facilitated. The mechanism of this protection by fat is obscure. It could be due to adsorption of the enzyme to the lipids of the fatty

TABLE II

PROTECTIVE EFFECT OF FAT TISSUE AND DIALYSIS ON SPLEEN PHOSPHOLIPASE A AGAINST HEAT TREATMENT

Treatments of spleen crude homogenate	*% Phospholipase A activity**	
	Without fat	*With fat*
No treatment (0 °C)	100	105
Dialysis against water (0 °C)	101	103
Heat treated, 65 °C/5 min, at pH 5.0	25	70
Dialysis against water (0 °C), and then heated, 65 °C/5 min, at pH 5.0	65	98

* Enzyme activity expressed in percentage, based on the total phospholipase A activity in the crude homogenate without treatment, and in the absence of surrounding fat, as 100%.

tissue, forming lipoprotein aggregates which are probably less sensitive to heat; a similar phenomenon was suggested by Sarda *et al.*[13] in their purification of a pancreatic lipase.

Dialysis against distilled water prior to heat treatment enhanced the recovery of spleen phospholipase activity without increasing the enzyme activity (Table II). This was unlike the results observed by De Haas *et al.*[14] where a concomitant increase of enzyme activity was found. It is likely that dialysis removes a low molecular weight substance(s) (possibly certain metal ions), the presence of which renders the enzyme more sensitive to heat denaturation.

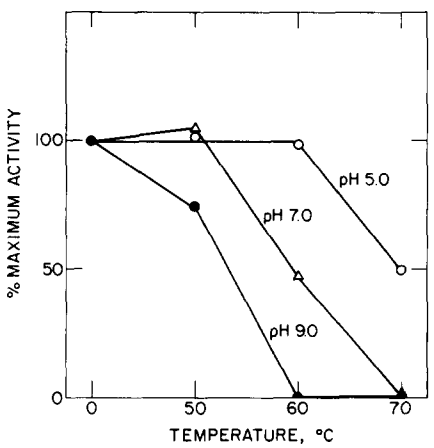

Fig. 5. Heat resistance of spleen phospholipase A in acidic medium.

Some functional properties of spleen phospholipase A

The purified spleen phospholipase A has an optimum pH around 7.0 (Fig. 6), though significant enzyme activity is still found at pH 6.0 and 9.0. Under the conditions used for determination of phospholipase activity, the hydrolytic reaction of the purified enzyme was linear for the first few min of incubation.

Ca^{2+} moderately activated the spleen phospholipase. A with a maximum activation at concentrations around 6–8 mM (Fig. 7). In the absence of calcium, unlike

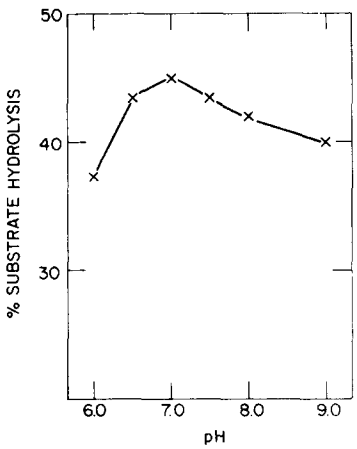

Fig. 6. Spleen phospholipase A activity as a function of pH.

the results obtained by De Haas *et al.*[14] with the pancreatic phospholipase, we consistently obtained about 55% of the maximum phospholipase activity. No satisfactory explanation can be offered at present. However, phospholipases with acidic pH optima have no requirement for Ca^{2+} (ref. 15), therefore, the existence of a trace amount of an acid phospholipase A in our enzyme preparation cannot be ruled out.

The following divalent cations: Mg^{2+}, Hg^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+} were inhibitory to the spleen phospholipase A (Fig. 7). With either phosphatidylcholine or phosphatidylethanolamine as substrate, at the concentration of 1 mM, EDTA inhibited the spleen phospholipase A completely (Fig. 8a), whereas *p*-chloromercuribenzoate, up to a concentration of $6 \cdot 10^{-4}$ M, had no significant effect on the enzyme activity (Fig. 8b). The spleen phospholipase A was completely inhibited by deoxycholate at a concentration of 0.05% when phosphatidylcholine was used as substrate;

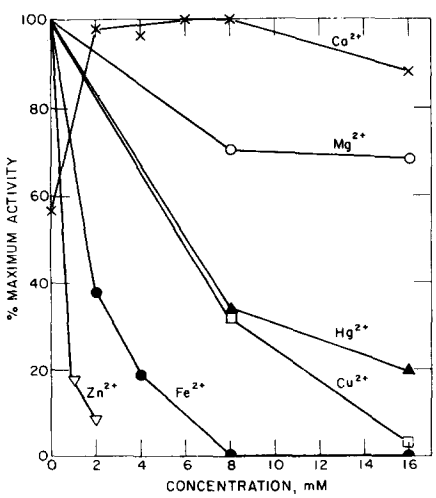

Fig. 7. Effect of various divalent cations on the spleen phospholipase A activity. 100% activity is that level of activity obtained under optimum assay conditions, *i.e.*, in the presence of 8 mM of $CaCl_2$.

there was, however, no significant inhibition when phosphatidylethanolamine was used (Fig. 8c). This selectivity is similar to that shown by human pancreatic phospholipase A (ref. 16). Triton X-100, a non-ionic detergent, inhibited the enzyme activity when either phosphatidylethanolamine or phosphatidylcholine was used as substrate (Fig. 8d). Phospholipases from snake venom or from spores of *Bacillus megaterium* do not show such sensitivity to detergents[17].

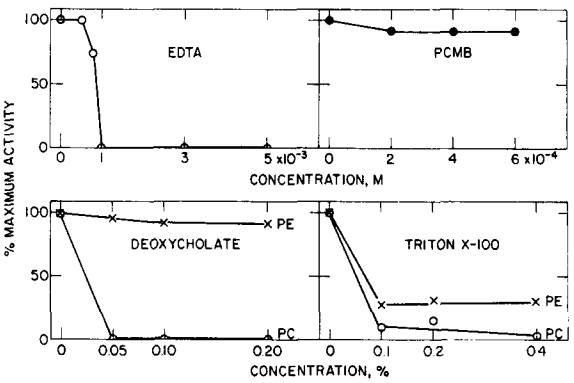

Fig. 8. a, Inhibitive effect of EDTA on spleen phopholipase A; b, Effect of *p*-chloromercuribenzoate on spleen phopholipase A; c, Effect of deoxycholate on spleen phospholipase A. ×—×, phosphatidyl ethanolamine was used as substrate; ○—○, phosphatidylcholine was used as substrate; d, Effect of Triton X-100 on spleen phospholipase A. ×—×, phosphatidylethanolamine was used as substrate; ○—○, phopshatidylcholine was used as substrate.

When 1-palmitoyl-[2-^{14}C]linoleoyl-glyceryl-3-phosphorylethanolamine was used as substrate for the purified spleen phospholipase A determination, no radioactive lysophosphatidylethanolamine was found as a degradative product, and all radioactivity was recovered as free fatty acids. Incubation of the purified spleen phospholipase A in the presence of [^{14}C]1-acylglycerylphosphorylethanolamine also did not yield detectable radioactive free fatty acids. The spleen phospholipase A seemed therefore specific for the fatty acid at the 2 position. However, the use of a doubly-labeled substrate, *e.g.* [1-^{3}H]palmitoyl-[2-^{14}C]linoleoylglyceryl-3-phosphorylethanolamine, would be desirable to ascertain such a specificity. Existence of two acid phospholipases, A_1 and A_2, in subcellular fractions of rat spleen has been recently reported[18]; the spleen phospholipase A under our study seems yet to be a third enzyme. It is conceivable that, similar to the liver, other tissues including the spleen, also have several different phospholipases; the enzymes with alkaline pH optima are generally membrane-bound, while those with acidic pH optima are soluble and occur only in lysosomes[10].

Comparing rat spleen and porcine pancreatic phospholipase A (ref. 14), the following points are observed: (1) both spleen and porcine pancreatic phospholipases A are specific for the fatty acid at the 2 position, (2) rat spleen phospholipase A has a lower pH optimum, (3) both rat spleen and porcine pancreatic phospholipases have an isoelectric point around 7.4, (4) both enzymes are inhibited by EDTA as well as by Hg^{2+} and Zn^{2+}, and (5) rat spleen phospholipase is inhibited by Mg^{2+}, Cu^{2+}, and Fe^{2+}, whereas the porcine pancreatic enzyme is insensitive to these ions.

The molecular weight of spleen phospholipase A

By use of Sephadex gel filtration, the molecular weight of spleen phospholipase A was estimated at 15 000 (Fig. 9). This value falls within the range of monomers of most phospholipases so far purified; they have molecular weights ranging between 13 000 and 20 000 (ref. 19).

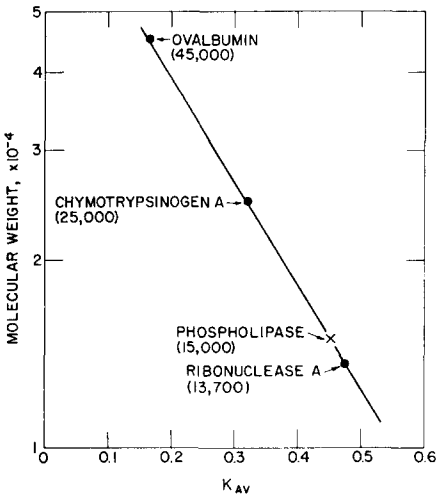

Fig. 9. Molecular weight determination of spleen phospholipase A by gel filtration on Sephadex G-75. Ovalbumin, chymotrypsinogen A and ribonuclease A with known molecular weights were used as reference proteins. $K_{av} = V_e - V_0/V_t - V_0$. V_e, elution volume for the protein; V_0, elution volume for Blue Dextran 2000 (Pharmacia); V_t, total bed volume of column.

General comments

Only phosphatidylethanolamine and phosphatidylcholine have been used as substrates in our present study of spleen phospholipase A; therefore, it is not clear whether this enzyme has a preference for phospholipids of specific charges. Substrates with stronger negative charge such as phosphatidylglycerol and phosphatidic acid and many others should be used to clarify this point.

During our purification procedure, we attempted to use the acidimetric assay described by Salach *et al.*[20] for the spleen phospholipase A, but unfortunately the method was not satisfactorily accurate for samples obtained at some of the purification steps. ^{14}C-labeled substrates were therefore used throughout our purification procedure. The drawbacks of this latter method are its tediousness and the high cost of labeled substrates needed at concentrations sufficiently high to produce zero-order kinetics. The determinations of the specific activity were therefore not satisfactorily accurate, hence a reliable estimation of the enzyme yield at various purification stages was difficult to obtain (Table I). After the final step of purification, several enzyme protein concentrations were incubated with the [^{14}C]phosphatidylethanolamine to ensure linear hydrolysis with the amount of enzyme added; however, this was not done after each purification step.

The existence of a phospholipase A with a pH optimum around 7.0 in calf spleen homogenate has been reported by Lloveras and Douste-Blazy[21], these same

authors have also partially purified an acidic phospholipase A from the calf spleen[22]. Our present study is the first attempt to purify a non-acidic, probably membrane-bound[10], phospholipase A from the spleen tissue. One of the major biological functions of the spleen is the removal of old and abnormal erythrocytes, and phospholipase A has recently been demonstrated to play an important role in the phenomenon of red cell membrane fragmentation[23,24]. The fragmentation of the red cell membrane might be an essential step leading to its subsequent removal by the splenic reticular cells. The present study not only demonstrates the existence of a non-acidic phospholipase A in rat spleen, it also demonstrates the feasibility of obtaining this enzyme in a purified form, which will enable us to investigate further the specific biological action of spleen phospholipase A on the red cell membranes *in vitro*.

ACKNOWLEDGMENT

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