# Isolation and characterization of a phospholipase $A_2$ from an inflammatory exudate

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**Abstract** Sterile peritoneal exudates produced in rabbits injected with 1% glycogen contain a phospholipase A activity in a cell-free supernatant fraction that hydrolyzed a synthetic phospholipid (1,2-diacyl-*sn*-glycero-3-phosphoethanolamine) and phospholipids of autoclaved *Escherichia coli*. This phospholipase activity (phosphatidylacylhydrolase EC 3.1.1.4) exhibited an apparent bimodal pH optimum (pH 6.0 and pH 7.5) and was $Ca^{2+}$-dependent; $Mg^{2+}$ and monovalent cations ($Na^+$ and $K^+$) did not substitute for $Ca^{2+}$ in the reaction; EDTA was a potent inhibitor. The phospholipase hydrolyzed 1-[1-$^{14}$C]palmitoyl-2-acyl-*sn*-glycero-3-phosphoethanolamine to form only radioactive lysophosphatidylethanolamine as the product, indicating that the enzyme had phospholipase $A_2$ specificity. The phospholipase $A_2$ was purified 302-fold by two successive chromatographic steps on carboxymethyl Sephadex. Gel filtration (Sephadex G75) of the purified enzyme resulted in a single peak of biological activity with a molecular weight of approximately 14,800. The same estimate of molecular weight was obtained by SDS–polyacrylamide gel electrophoresis, which yielded a single band. Polyacrylamide gel electrophoresis of this fraction at pH 4.3 revealed a single protein band migrating beyond lysozyme, with the dye front, suggesting that this protein was more basic than lysozyme (pI 10.5). The enzymatic and physical-chemical characteristics of this soluble enzyme were remarkably similar to a recently described phospholipase $A_2$ of rabbit polymorphonuclear leukocytes derived from glycogen-induced peritoneal exudates. The possible origin and physiological role of this soluble enzyme are discussed.

**Supplementary key words** phospholipid · $Ca^{2+}$ dependence · positional specificity · molecular size · cationic protein · polymorphonuclear leukocyte · serum

Phospholipases (PLA) accumulate in the peritoneal fluid of patients with cirrhosis and acute pancreatitis (1). PLA reaction products, in particular lysophospholipids, are powerful cytotoxic substances and produce intense inflammation when applied locally (2) or intraperitoneally (3, 4). Furthermore, it has been shown that a variety of commonly used adjuvants increase lysophospholipid production in areas of inflammation (5), and that lysophospholipase injected into an inflamed site has potent anti-inflammatory activity, presumably due to its ability to degrade lysophospholipid (5). These data suggest a possible role for PLA in the pathogenesis of inflammatory diseases; but at this time little is known concerning the appearance, origin, or characteristics of PLA in inflammatory fluids.

To begin to study the possible role of PLA in the inflammatory process, we have purified and characterized a cell-free $PLA_2$ from rabbit peritoneal fluid. This enzyme closely resembles the granule-associated $PLA_2$ of polymorphonuclear leukocytes found in rabbit peritoneal exudates that we have recently described (6, 7).

## MATERIALS AND METHODS

### Labeled substrates

Phospholipids of *Escherichia coli* W were labeled with [1-$^{14}$C]oleate as previously described (6). Generally, 95% of the incorporated label was in phospholipid, and the distribution of the label closely corresponded to the chemical composition of *E. coli* phospholipid, i.e., phosphatidylethanolamine, 50–65%; phosphatidylglycerol, 15–20%; and cardiolipin, 10–15%. Radiolabeled *E. coli* was autoclaved for 15 min at 120°C and 2.7 kg/cm². As shown by snake venom hydrolysis (*Crotalus adamanteus*), 95% of the [1-$^{14}$C]oleate was in the 2-acyl position of the phospholipids.

The biosynthetic preparation of 1-[1-$^{14}$C]palmitoyl-2-acyl-*sn*-glycero-3-phosphoethanolamine was carried out as previously described by Waite and Van Deenen (8).

Abbreviations: PLA, phospholipase A; PMN, polymorphonuclear leukocyte; SDS, sodium dodecyl sulfate; CM, carboxymethyl; PAGE, polyacrylamide gel electrophoresis; PSF, peritoneal supernatant fluid

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### Phospholipase assay

Phospholipase $A_1$ and $A_2$ specifically hydrolyze the fatty ester linkage in the C-1 and C-2 positions of phospholipids, respectively, to yield free fatty acid and lysophospholipid. Using *E. coli* labeled with [1-$^{14}$C]oleate as substrate, reaction mixtures in a total volume of 0.5 ml contained (unless otherwise stated) $2.5 \times 10^8$ cells ([1-$^{14}$C]oleate-labeled *E. coli*, representing approximately 5 nmol of phospholipid, and 4000 cpm), 100 mM Tris-HCl buffer, pH 7.5, 10 mM $CaCl_2$, and 5–10 $\mu$g of protein. Reaction mixtures were incubated for 4 min at 37°C. Reactions were stopped by the addition of two volumes of methanol and the products of hydrolysis were extracted by the method of Bligh and Dyer (9). Radioactive lipids were separated by thin-layer chromatography on precoated silica gel plates (EM Reagents Elmsford, NY) developed in petroleum ether–ethyl ether–acetic acid 80:20:1 (v/v). Chromatograms were stained with $I_2$ vapor and the silica gel containing radioactive lipid was scraped into vials containing scintillation fluid. Radioactivity was determined in a Beckman LS-333 scintillation counting system. When pure phosphatidylethanolamine was used as substrate, reaction mixtures in a total volume of 1.0 ml contained 400 nmol of 1-[1-$^{14}$C]-palmitoyl-2-acyl-*sn*-glycero-3-phosphoethanolamine

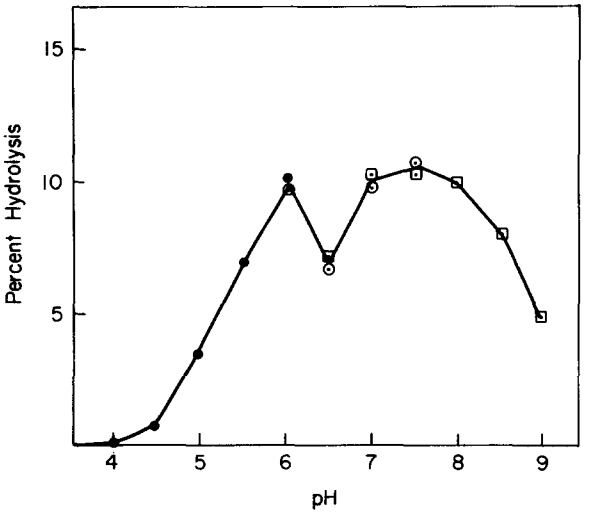


**Fig. 1.** Effect of pH on the hydrolysis of phospholipid in [1-$^{14}$C]oleate-labeled, autoclaved *E. coli* by peritoneal supernatant fluid. The incubation conditions are described in Materials and Methods, except for the variation in pH. The buffers used were sodium acetate, pH 3.5–6.0 (● —— ●); Tris–maleate, pH 6.0–7.5 (⊙ —— ⊙); and Tris-HCl, pH 6.5–9.0 (⊡ —— ⊡). Incubations were for 10 min. in the presence of 50 $\mu$g of protein (PSF). Phospholipase A activity (1-$^{14}$C-labeled free fatty acid released from [1-$^{14}$C]oleate-labeled *E. coli*) was calculated as the percentage of the total *E. coli* lipid radioactivity and can be converted to nmol of free fatty acid released by assuming that $2.5 \times 10^8$ *E. coli* cells contain 5 nmol of phospholipid (6).

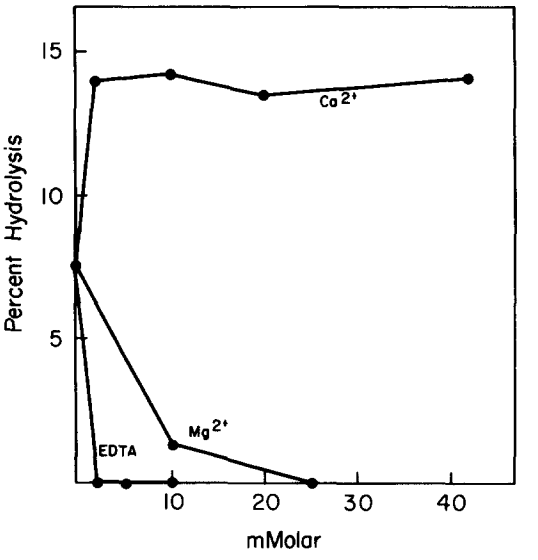


**Fig. 2.** Calcium requirement for phospholipase A activity. Different concentrations of $CaCl_2$, $MgCl_2$, or EDTA were added to the standard reaction mixtures containing 50 $\mu$g of protein in peritoneal supernatant fluid (PSF). NaCl and KCl (tested at concentrations up to 500 mM—not shown) produced PLA inhibition that was identical to the $MgCl_2$ effect shown.

(10,000 cpm added as an ultrasonic suspension in water), 100 mM Tris-HCl buffer, pH 7.5, 10 mM $CaCl_2$, and protein as indicated. Reaction mixtures were incubated for 30 min at 37°C; the reaction was stopped with two volumes of methanol and lipids were extracted by the method of Bligh and Dyer (9). Radioactive lipids were separated by thin-layer chromatography on Silica Gel G plates which were first developed in chloroform–petroleum ether (bp 63–75°C)–acetic acid 70:30:4 (v/v), and then in chloroform–methanol–water 70:30:4 (v/v). Radioactive lipids were isolated for scintillation counting as described above.

### Purification of peritoneal fluid phospholipase A

Overnight sterile peritoneal exudates (250 ml/rabbit) were produced in rabbits by injection of glycogen in physiological saline (6). The polymorphonuclear leukocytes (95% of the cells as judged by differential cell count) were sedimented by centrifugation at 5000 *g* for 10 min at 4°C. The cell-free, 5000 *g* peritoneal supernatant fluid (PSF) was brought to a final concentration of 0.5 M NaCl by the addition of ice-cold 4.0 M NaCl. Thereafter all steps were done at 4°C. The 0.5 M NaCl–PSF was applied to a CM–Sephadex chromatography column (2.5 × 20 cm) equilibrated in 10 mM Tris-HCl buffer, pH 7.5, and 0.5 M NaCl, and was eluted by 10 column volumes of buffered 0.5 M NaCl and two column volumes of buffered 3.0 M NaCl to yield a 0.5–3.0 M NaCl–CM fraction (CM-I) enriched in

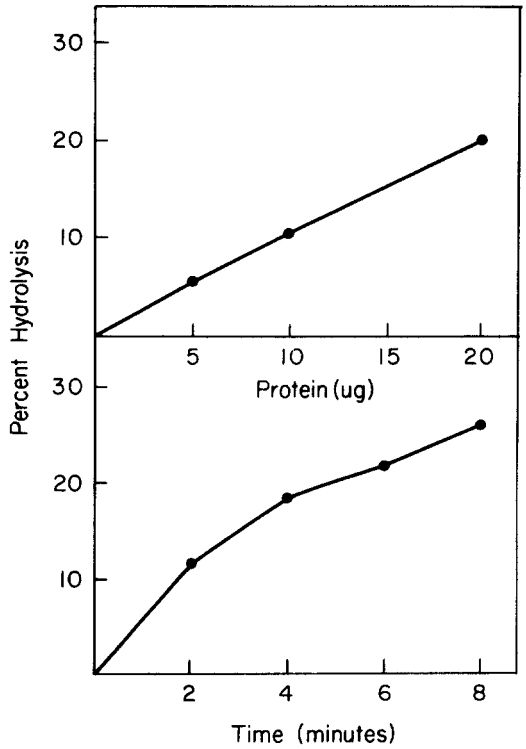


**Fig. 3.** Effect of time and protein concentrations on phospholipase A activity. Otherwise standard reaction mixtures were incubated for 2 min with increasing amounts of CM-I fraction (see Table 1), or for time studies with 10 $\mu$g of CM-I fraction.

PLA activity. CM-I was subsequently dialyzed to adjust the buffered salt concentration to 0.2 M NaCl; it was then reapplied to a buffered CM–Sephadex column (2.5 × 20 cm) equilibrated in 0.2 M NaCl and washed with three column volumes of buffered 0.2 M NaCl. Thereafter, a total of 200 ml of a continuous NaCl gradient from 0.2 M to 4.0 M NaCl was applied and 35 fractions (5.5 ml/fraction) were collected. Fractions 11–18 were pooled and designated CM-II. Aliquots of CM-II were chromatographed on Sephadex G-75, subjected to SDS–PAGE by the method of Weber and Osborn (10), and PAGE at pH 4.3 by the method of Reisfield (11). Polyacrylamide gels were stained with Coomasie Brilliant Blue G (12) and were destained and stored in 7% acetic acid. Protein was determined by the method of Lowry et al. (13) using BSA as standard, or by measuring the absorbance at 260 and 280 nm (14). Sodium chloride concentrations were determined by atomic absorption spectroscopy.

## RESULTS

### General properties of PLA

The pH for optimal phospholipase A activity in the 5000 *g* supernatant fluid of peritoneal exudates is shown in **Fig. 1.** With the exception of a reproducible decrease in activity at pH 6.5, maximal PLA activity was apparent over a broad pH range (pH 6–8). No accumulation of radioactivity lysocompounds was detected at any pH.

**Figs. 2** and **3** depict optimal conditions of time, protein, and divalent cation requirement for phospholipid hydrolysis at pH 7.5. PSF–PLA required $Ca^{2+}$ for optimal activity; $Mg^{2+}$, and $Na^+$ or $K^+$ in concentrations up to 500 mM (not shown) inhibited activity, and no activity was found in the presence of EDTA (Fig. 2). PLA activity of PSF and purified CM fractions was consistently linear with respect to time and protein content when phospholipid hydrolysis of $2.5 \times 10^8$ autoclaved *E. coli* cells was less than 20% (Fig. 3). The considerable deviation from linearity that occurred when the hydrolysis was greater than 20% is probably due to limited accessibility of phospholipid within the *E. coli* envelope, which remains largely intact despite autoclaving. Because incubations employing [1-$^{14}$C]oleate-labeled, autoclaved *E. coli* in which 95% of the label was in the 2-position of the bacterial phospholipid yielded only radioactive fatty acid as product, it appears that the phospholipase was of $A_2$ specificity. However, the accumulation of this product could have been due to the combined effects of phospholipase $A_1$ and lysophospholipase activities.

To demonstrate the positional specificity of the peritoneal PLA more conclusively, PSF and the purified CMI fraction were incubated with 1-[1-$^{14}$C]palmitoyl-

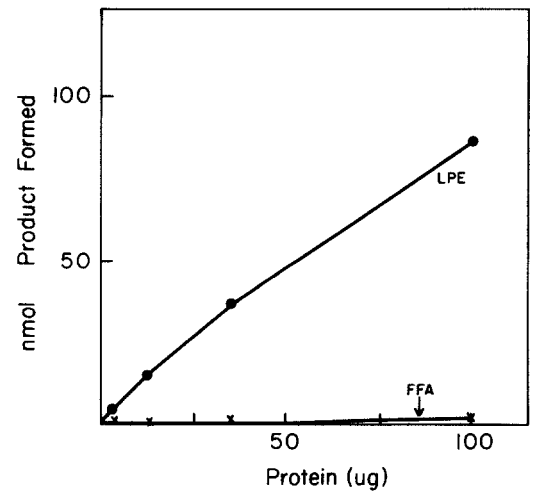


**Fig. 4.** Positional specificity of phospholipase A. Reaction mixtures as described in Materials and Methods contained 400 nmol of 1-[1-$^{14}$C]palmitoyl-2-acyl-*sn*-glycero-3-phosphoethanolamine and increasing concentrations of CM-I, and they were incubated for 30 min. Phospholipase A activity is expressed as nmol of product formed per 30 min: [$^{14}$C]lysophosphatidylethanolamine (LPE ● —— ●) and $^{14}$C-labeled free fatty acid (FFA X —— X). Similar results were obtained with the CM-II fraction.

TABLE 1. Purification of phospholipase $A_2$ from peritoneal fluid

| Fraction | Total Protein | Phospholipase Activity[a] Total Units | Recovery (%) | Units/mg | Fold Purification |
|---|---|---|---|---|---|
| | *mg* | | | | |
| Peritoneal fluid | 560 | $5.8 \times 10^4$ | 100 | 103 | |
| CM-I | 11.8 | $1.7 \times 10^4$ | 29 | 1,440 | 14 |
| CM-II | 0.45 | $1.4 \times 10^4$ | 24 | 31,111 | 302 |

[a] PLA activity in units of nmol free fatty acid/hr.

The enzyme was purified as described in the text and assayed using radiolabeled, autoclaved *E. coli* as substrate. Reaction mixtures were incubated for 2 and 4 min with three different protein concentrations at each time point. Only those points in the linear range of hydrolysis vs. time and protein were averaged to quantitate PLA activity.

2-acyl-*sn*-glycero-3-phosphoethanolamine (**Fig. 4**). Only radioactive lysophosphatidylethanolamine accumulated as product, indicating that the PLA in the ascitic fluid specifically hydrolyzed the 2-acyl ester bond of phospholipids. Glycerophosphorylcholine was hydrolyzed by the CM-I fraction at one-half the rate of glycerophosphorylethanolamine (400 nmol/hr per mg); whereas autoclaved *E. coli* phospholipid was hydrolyzed at an even greater rate (1401 nmol/hr per mg, **Table 1**). Because of the increased sensitivity and economy, autoclaved *E. coli* was used as substrate in this study.

### Purification of peritoneal fluid $PLA_2$

PSF–$PLA_2$ was purified 302-fold in a yield of 24% by two successive steps of CM–Sephadex chromatography (Table 1). PSF was adjusted to 0.5 M NaCl before chromatography in order to achieve reproducible elution patterns (**Fig. 5**). Purified $PLA_2$ was eluted in a continuous salt gradient between 1.0 M and 2.0 M NaCl, yielding a discrete peak of activity at 1.5 M NaCl. When Folin protein determinations were performed on fractions 11–18, the sp act of the PLA was constant (approx. 31,000 units/mg) and, therefore, these fractions were pooled (CM-II). CM-II was stable when stored in the presence of 1.5 M NaCl for at least 6 weeks at 4°C. Freezing of CM-II or removal of the NaCl by dialysis or ultrafiltration resulted in rapid loss of enzymatic activity. An aliquot of CM-II was chromatographed on a calibrated Sephadex G-75 column equilibrated with buffered 1.5 M NaCl (**Fig. 6**). The high salt concentration was necessary to prevent adsorption and apparent protein aggregation. Ninety percent of the $PLA_2$ activity was recovered in a single symmetrical peak corresponding to a mol wt of 14,800. CM-II migrated as a single band in SDS–PAGE with an apparent mol wt of 14,700 (**Fig. 7**).

**Fig. 8** shows the electrophoretic behavior of PSF, CM-II, and lysozyme at pH 4.3.PSF (1 mg) contained primarily slow-moving proteins ($R_f < 0.5$) and two fast-moving cationic species; one appeared at the indicator dye front and a second faint band appeared 0.5 cm from the dye front and corresponded to the lysozyme standard. CM-II (50 $\mu$g) migrated as a single band at the dye front beyond the lysozyme standard.

## DISCUSSION

Recent studies by Thouvenot, Durand, and Douste-Blazy (1) demonstrated the presence of phospholi-

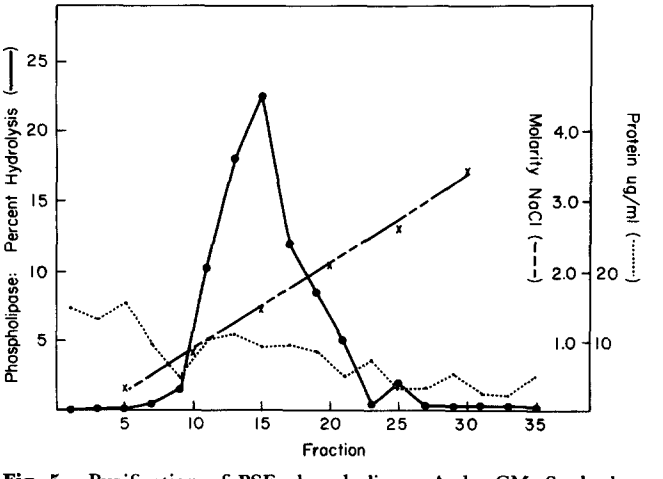


**Fig. 5.** Purification of PSF phospholipase $A_2$ by CM–Sephadex chromatography. Two hundred ml of the CM-I fraction was applied to a CM–Sephadex column (2.5 × 20 cm) and eluted (70 ml/hr) as described in Materials and Methods. Standard reaction mixtures for the measurement of PLA activity contained 5 $\mu$l aliquots of each fraction and were incubated for 4 min. In seven such preparations the recovery of $PLA_2$ activity ranged from 70 to 88%. Protein was estimated by absorbance at 260 and 280 nm (14).

pase A activity in ascitic fluids by measuring hydrolysis of [$^{32}$P]phosphatidylethanolamine; lysophosphatidylethanolamine accumulated at pH 7.2 in the presence of deoxycholate (1 mg). These authors found elevated PLA activities in peritoneal fluids from patients with cirrhosis and acute pancreatitis. However, the characteristics of the enzyme(s) that hydrolyzes phospholipid in ascitic fluids were not further defined.

We now describe the initial isolation and characterization of a soluble, $Ca^{2+}$-dependent phospholipase $A_2$ derived from glycogen-induced rabbit peritoneal exudates. This enzyme was maximally active over a broad pH range and, at this stage of purification, its apparent mol wt is approximately 14,800 as judged by gel filtration and SDS–PAGE. Thus, it is similar in size to well characterized $PLA_2$ from pancreas (15), various snake venoms (16), and bee venom (17). The peritoneal $PLA_2$ migrated in PAGE at pH 4.3 with the indicator dye front, 0.5 cm beyond lysozyme. Because migration in polyacrylamide gels under these conditions is a function of both molecular size and charge, and since the $PLA_2$ and lysozyme have almost identical molecular weights, it appears that this $PLA_2$ is a more basic protein than lysozyme (pI 10.5). This characteristic may contribute to its tendency to adsorb and aggregate in the absence of high salt concentrations. The unusual cationic charge of the peritoneal $PLA_2$ is of particular interest because of recent reports that suggest that basic $PLA_2$ may be more cytotoxic than anionic $PLA_2$ (7, 17–19). Thus, the basic $PLA_2$ from *Agrokistron* (18) and *Vipera beris* (19) snake venoms are cytotoxic and increase capillary permeability; but the acidic $PLA_2$ isolated from the same venoms are inactive in this regard. Well-characterized $PLA_2$ with highly basic isoelectric points (pI > 10) have been described in the pig pancreas (15) and bee venom (17).

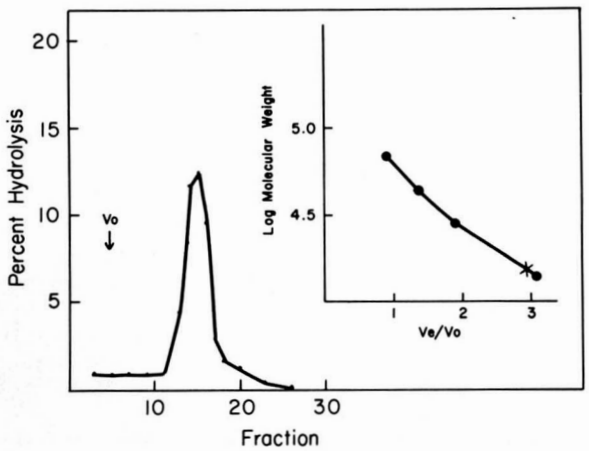


**Fig. 6.** Gel filtration of CM-II on Sephadex G-75. CM-II (1.5 ml, 10 μg/ml) was applied to a Sephadex G-75 column (2.0 × 40 cm) equilibrated with buffered 1.5 M NaCl. For phospholipase activity, standard incubation conditions were used except that 0.2 ml of each column fraction was incubated with substrate for 90 min; PLA activity is expressed as percent hydrolysis per 0.2-ml aliquot per 90 min. The recovery of total PLA activity was 90%. For estimation of mol wt the column was equilibrated in buffered 1.5 M NaCl and calibrated with standard proteins (from top left to lower right: bovine serum albumin, ovalbumin, α-chymotrypsinogen, and ribonuclease $A_1$).

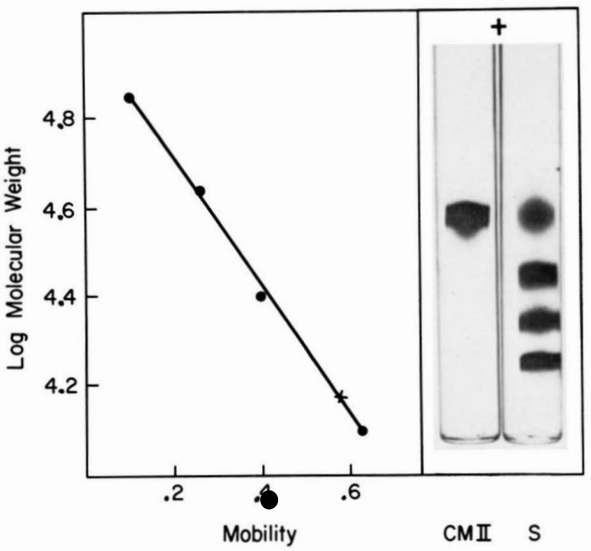


**Fig. 7.** Estimate of mol wt by SDS–PAGE. The CM-II fraction (20 μg) was applied to 7% polyacrylamide gels; 15 μg of each standard was applied (from top left to lower right: bovine serum albumin, ovalbumin, α-chymotrypsinogen, and ribonuclease $A_1$) and gels were electrophoresed at 6 mA/gel for 5 hr.

We have recently described the granule localization of a membrane-associated $PLA_2$ in PMN (6), and its properties are similar to this soluble enzyme with respect to positional specificity, $Ca^{2+}$ requirement, and pH optima. Initial purification of the PMN $PLA_2$ by CM–Sephadex chromatography (7) and gel electrophoresis at pH 4.3[3] indicate that the PMN–$PLA_2$ is also a protein with an approximate mol wt of 15,000 that appears more cationic than lysozyme (7). Studies are underway to further compare the properties of these two phospholipase $A_2$ activities.

Intraperitoneal injection of glycogen in isotonic saline produces an inflammatory response in rabbits resulting in the migration of serum proteins and blood cell elements into the peritoneum (7). Eight to ten hours after injection, 95% of the cells in the exudate are polymorphonuclear leukocytes. Because of the striking similarity between the enzymatic and physical properties of the peritoneal $PLA_2$ and the PMN–$PLA_2$, the exudate enzyme may be derived

[3] Franson, R., J. Weiss, and P. Elsbach. Unpublished data.

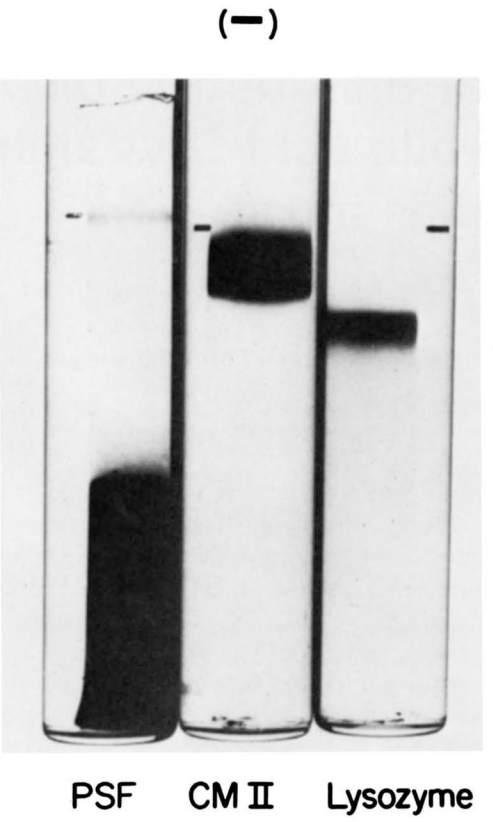


**Fig. 8.** Electrophoresis of CM-II at pH 4.3. PSF (1 mg), CM-II (50 $\mu$g), and lysozyme (25 $\mu$g) were applied to 7% polyacrylamide gels and gels were electrophoresed at 4 mA/gel for 2 hr. The solid line (——) marks the migration of the leading edge of the tracking dye, methyl green.

from polymorphonuclear leukocytes that "turn over" rapidly in areas of inflammation. Alternatively the PSF–$PLA_2$ could be a serum-derived component since mammalian sera also contain phospholipase $A_2$ activity; however, preliminary experiments indicate that the properties of the rabbit serum phospholipase $A_2$ differ from those described for the $PLA_2$ of ascitic fluid and PMN leukocytes. Therefore, it appears that the soluble PLA is probably of leukocyte origin.

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