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PHOSPHOLIPASE A_1 ACTIVITY OF GUINEA PIG PANCREAS

D. A. WHITE*, D. J. POUNDER AND J. N. HAWTHORNE**
Department of Biochemistry, University of Birmingham, Birmingham (Great Britain)

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SUMMARY

1. Freshly prepared homogenates of guinea pig pancreas contain high phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) activity.

2. Using 1-([9,10-$^3H_2$]stearoyl)phosphatidylcholine, 2-(1-^{14}C]linoleyl)phosphatidylcholine and [^{32}P]phosphatidylinositol two pH optima were found at pH 6.0 and pH 8.5.

3. The activity at pH 6.0 was due initially to phospholipase A_1, followed by lysophospholipase activity.

4. Kinetic studies on the activity at pH 6.0 showed that phosphatidylcholine and phosphatidylinositol were good substrates and the latter had a v_{max} of 129 μmoles substrate hydrolysed per mg protein per h.

5. Addition of Ca^{2+} to the assay system inhibited the activity. EDTA had no effect.

INTRODUCTION

WHITE AND HAWTHORNE[1] have recently shown that during fractionation of guinea pig pancreas slices which had been previously labelled with [^{32}P]orthophosphate, much of the radioactivity which was associated with phosphatidylinositol in the homogenate appeared as lysophosphatidylinositol on examination of the subcellular fractions. Apparently a phospholipase A had been active during fractionation. The phospholipase A activity of horse and rat pancreas is described by VAN DEN BOSCH *et al.*[2]. These authors showed that at pH 7.4 both phospholipase A_1 and A_2 activities were present. We now report some of the properties of the A_1 enzyme of fresh guinea pig pancreas homogenates and fractions.

MATERIALS AND METHODS

Animals

The pancreas was removed from each guinea pig immediately after killing and dissected free of fat and connective tissue prior to homogenization or slicing.

* Present address: Department of Physiological Chemistry, Johns Hopkins Medical School, Baltimore, Md. 21205, U.S.A.

** Present address: Department of Chemistry, University of Calgary, Calgary 44, Alberta, Canada.

Materials

[^{32}P]Orthophosphate (PBSI) was obtained from The Radiochemical Centre (Amersham, Bucks, U.K.). *Crotalus adamanteus* venom phospholipase A_2 was purchased from Koch-Light Limited (Colnbrook, Bucks, U.K.). Phosphatidylcholine and phosphatidylinositol were prepared from ox brain as described by ANSELL AND HAWTHORNE[3].

Analytical methods

Protein was determined by the method of LOWRY *et al.*[4]. The method of BARTLETT[5] as modified by GALLIARD *et al.*[6] was used for determination of phosphorus.

Scintillation fluid. The basic phosphor contained 6 g 2,5-diphenyloxazole and 0.12 g 1,4-bis(5-phenyloxazolyl)benzene per l of toluene. For counting samples from thin-layer chromatograms this was diluted with Triton X-100 (phosphor:Triton, 2:1, v/v).

Preparation of 2-([1-^{14}C]linoleyl)phosphatidylcholine. For the introduction of [1-^{14}C]linoleic acid into the 2-position of phosphatidylcholine, lysolecithin was prepared from ox brain lecithin using *Crotalus adamanteus* venom (LONG AND PENNY)[7] and the lysolecithin was reacylated by the biosynthetic method of ROBERTSON AND LANDS[8]. The radioactive phospholipid was isolated by thin-layer chromatography on Silica gel H (Camag, Muttenz, Switzerland) using the system of SKIPSKI *et al.*[9]. The labelled phosphatidylcholine prepared in this way had a specific activity of 16 000 counts/min per μg lipid P and, as shown by analysis of the liberated fatty acid following hydrolysis by *Crotalus adamanteus* venom, more than 99% of the activity was in the C-2 position of the glycerol moiety.

The authors are indebted to Dr. H. Van den Bosch for a sample of 1-([9,10-$^{3}H_2$]-stearoyl)phosphatidylcholine. The sample was purified by deacylating with snake venom as above and reacylating with unlabelled linoleic acid.

Preparation of [^{32}P]phosphatidylinositol. Slices from one or two freshly excised guinea pig pancreases were incubated in 10 ml bicarbonate buffer (KREBS AND HENSELEIT)[10] containing 2 mC [^{32}P]orthophosphate (carrier-free), 10^{-4} M acetylcholine and 10^{-4} M eserine. The slices were kept in motion by bubbling O_2–CO_2 (95:5, v/v) through the medium during the 2-h incubation. The slices were then rapidly filtered through glass wool and washed with cold NaCl (0.9%) to remove water-soluble radioactivity prior to homogenization in a Waring blendor in 40 ml of chloroform–methanol (2:1, v/v). 8 ml of distilled water was added to the homogenate to give two phases which after centrifugation were separated by a protein layer. The lower phase was evaporated to dryness and the residue was dissolved in 3 ml of chloroform. Phosphatidylinositol was isolated from the extract by preparative thin-layer chromatography using the system of SKIPSKI *et al.*[9]. The area on the plate corresponding to phosphatidylinositol was extracted with chloroform–methanol (1:2, v/v) and the extract taken to dryness prior to being dissolved in a known volume of chloroform. Samples of this were assayed for specific radioactivity. Generally extracts from two pancreases yielded about 50 μg phosphatidylinositol phosphorus of total activity $1 \cdot 10^6$–$1.5 \cdot 10^6$ counts/100 sec. This was diluted with ox brain phosphatidylinositol for use as substrate in the phospholipase assays.

Preparation of glycerylphosphorylinositol. A sample of ox brain phosphatidylinositol was deacylated by the method of DAWSON[11] and glycerylphosphorylinositol was

purified on a column of Dowex 50w (pyridinium form). The final eluate was taken to dryness under reduced pressure and dissolved in a known volume of water. The purity of the sample was checked by paper chromatography[11].

Assay of phospholipase A activity. The basic system (total volume 1 ml) contained phosphatidylcholine 7 μg P, 20 mM maleate buffer (pH 6.0) and enzyme protein. Incubations were usually for 10 min at 37°, the reaction being started by the addition of substrate in an emulsion after 1 min pre-incubation of the system. The reaction was stopped with chloroform–methanol (1:2, v/v) and washed by the procedure of GARBUS *et al.*[12], as modified by PROTTEY AND HAWTHORNE[13] for incubation volumes of 1 ml. Carrier lysophosphatidylcholine was added to the lower phase which was then taken to dryness under nitrogen and the lipid extract was re-dissolved in 0.3 ml chloroform–methanol (2:1, v/v). Samples (0.1 ml) of this were put on to thin-layer plates and developed according to SKIPSKI *et al.*[9]. The areas on the developed chromatogram corresponding to lysophosphatidylcholine, phosphatidylcholine and free fatty acid were scraped off into counting vials and 0.5 ml water, 0.5 ml methanol and 9 ml phosphor were added before counting in a Nuclear-Chicago liquid scintillation counter.

For the assay using phosphatidylinositol as substrate the system was essentially the same. However, after the reaction had been stopped with chloroform–methanol, lysophosphatidylinositol could be made to partition into either the aqueous phase or organic phase by using either distilled water or a 0.1 M $CaCl_2$ solution, respectively, to effect phase separation[14]. Using the "water wash", lysophosphatidylinositol partitions into the aqueous phase, while using the "calcium wash" it partitions into the lower organic phase. Use was made of this to determine the amount of lysophosphatidylinositol present after hydrolysis, by the difference in counts in the upper phase following such washes.

Identification of the products of the enzymatic hydrolysis of phosphatidylinositol at pH 6.0. In order to identify the products of the phospholipase A assay a large-scale incubation containing homogenate protein (3 mg), [^{32}P]phosphatidylinositol (30 μg lipid P, 200 000 counts/100 sec), maleate buffer pH 6.0 (20 mM) in a final volume of 1 ml was incubated at 37° for 10 min. The reaction was stopped with chloroform–methanol (1:2, v/v) as previously described and the phases were separated with 20 mM $CaCl_2$. The upper phase was passed down a Dowex-50 (pyridinium form) column (1 cm × 10 cm), non-radioactive glycerylphosphorylinositol (200 μg P) was added to the eluate and this solution was applied to a Dowex-1 (formate) column (1 cm × 15 cm) and eluted with borate–formate as described by HÜBSCHER AND HAWTHORNE[15] and HÜBSCHER *et al.*[16]. Confirmation of the identity of the peaks obtained from the column was furnished by chromatography using the system of DAWSON[11]. The lower phase from the assay was evaporated to dryness, re-dissolved in 0.2 ml chloroform and examined by thin-layer chromatography using the system of SKIPSKI *et al.*[9]. Autoradiographs of the developed plate were made.

Fractionation of guinea pig pancreas. The simple fractionation scheme of PROTTEY AND HAWTHORNE[13] was used to give nuclear, mixed pellet, microsomal and supernatant fractions.

RESULTS

Effect of pH on phospholipase A activity

The pH curves using 2-([1-^{14}C]linoleyl)phosphatidylcholine, 1-([9,10-$^{3}H_2$]-stearoyl)phosphatidylcholine and [^{32}P]phosphatidylinositol all indicated an optimum phospholipase A activity at pH 6 (Figs. 1–3). Early experiments with larger amounts of protein (1 mg) in the assay system for phosphatidylcholine indicated a second pH optimum at pH 8.5 (Fig. 1) but this peak was not apparent at low protein concen-

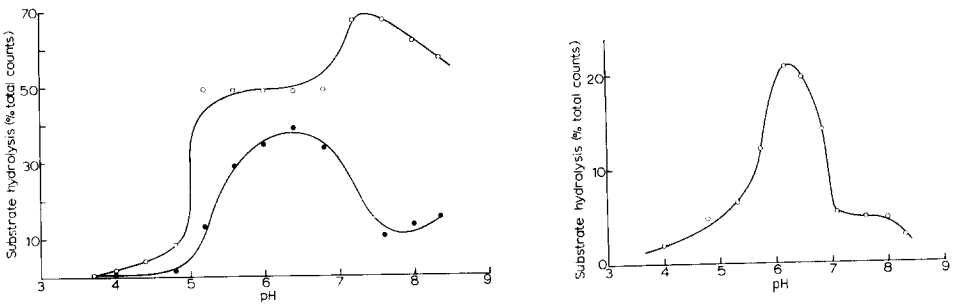


Fig. 1. pH curve of hydrolysis of 2-([1-^{14}C]linoleyl)phosphatidylcholine. ○—○, total phospholipase A hydrolysis measured by the decrease in counts in phosphatidylcholine; ●—●, minimal phospholipase A_1 activity measured by increase in counts in lysophosphatidylcholine.

Fig. 2. pH curve of hydrolysis of 1-([9,10-$^{3}H_2$)stearoyl)phosphatidylcholine measured by the increase in counts in free fatty acid.

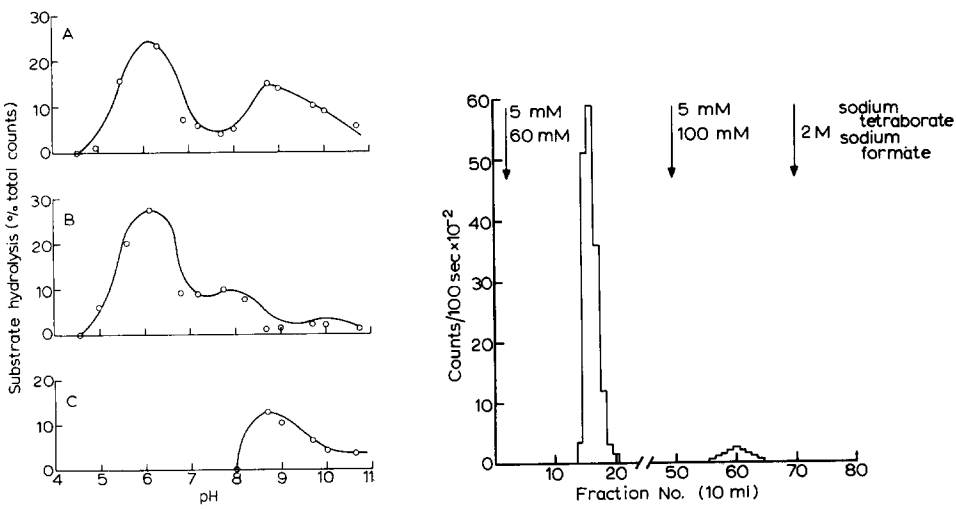


Fig. 3. pH curve of hydrolysis of [^{32}P]phosphatidylinositol. A. Total hydrolysis measured from the percentage of counts in upper phase following a "water wash" (see MATERIALS AND METHODS). B. Combined phospholipase A + lysophospholipase activity obtained from counts in upper phase following a "calcium wash", *i.e.* counts due to glycerylphosphorylinositol. C. The difference between A and B due to lysophosphatidylinositol produced by phospholipase A alone.

Fig. 4. Products of reaction at pH 6.0 using phosphatidylinositol as substrate.

trations (Fig. 2). With phosphatidylinositol as substrate both peaks were given at low protein concentration (Fig. 3). The major products of the reaction when [^{32}P]-phosphatidylinositol was substrate were shown to differ at the two pH optima. At pH 8.5 the major product of the enzyme action was lysophosphatidylinositol, as shown by the two wash procedures and also by autoradiography of the thin-layer chromatograms of the lower phase following a "calcium wash". Two spots appeared on the autoradiogram; unchanged phosphatidylinositol and lysophosphatidylinositol. Less than 5% of the radioactivity was associated with glycerylphosphorylinositol at this pH. At pH 6.0 glycerylphosphorylinositol was the major product detected (> 95% total counts released) by column chromatography (Fig. 4). The eluted counts followed closely the elution of phosphate from the column. Some inositol phosphate (< 2% total counts) was seen after hydrolysis. The appearance of glycerylphosphorylinositol as the main product indicated the presence of a lysophospholipase of activity at least equal to the initial phospholipase A activity.

When 2-([1-^{14}C]linoleyl)phosphatidylcholine was used as substrate for assay at pH 6.5, much of the activity was retained in lysophosphatidylcholine (Fig. 1), indicating phospholipase A_1 activity. By analogy with experiments when phosphatidylinositol was substrate, much of the ^{14}C found in fatty acid following hydrolysis may be derived from lysophospholipase activity on 2-([1-^{14}C]linoleyl)lysophosphatidylcholine first formed and not from phospholipase A_2 activity. Evidence that counts found in fatty acid are not derived from phospholipase A_2 activity comes from hydrolysis using 1-([9,10-$^3H_2$]stearoyl)phosphatidylcholine as substrate. Less than 0.5% of the counts were found in lysophosphatidylcholine which would be the major labelled product of phospholipase A_2 action. Inclusion of EDTA, an inhibitor of pancreatic phospholipase A_2[18,19] had no inhibitory effect on hydrolysis (Fig. 7), which further indicates that phospholipase A_2 was not contributing to the observed hydrolysis under the experimental conditions.

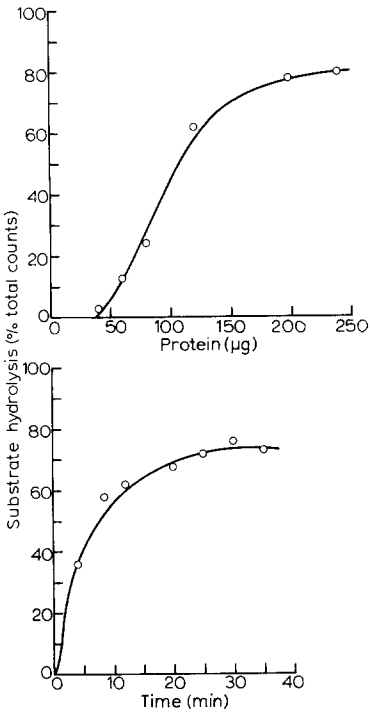
Using 2-(1-^{14}C]linoleyl)phosphatidylcholine as substrate, counts measured in lysophosphatidylcholine are derived from and represent a minimal value of true phospholipase A_1 activity. Total phospholipase A activity is measured as the sum of the activities indicated by counts in lysophosphatidylcholine and free fatty acid following hydrolysis.

Kinetics of enzyme activity

The time and protein concentration curves (Figs. 5 and 6) indicate that hydrolysis is rapid and under saturating conditions the rate of hydrolysis of phosphatidylinositol is of the order of six times that of phosphatidylcholine. In experiments where phosphatidylinositol was substrate total hydrolysis was measured as the percentage of counts in the upper phase following a "water wash", *i.e.* counts due to glycerylphosphorylinositol and lysophosphatidylinositol. The contribution from lysophosphatidylinositol was measured as the difference in counts between the upper phase following "water and calcium washes".

Effect of Ca^{2+} on activity

Addition of Ca^{2+} to the incubation medium inhibited phospholipase A_1 activity. The effect of increasing concentrations of Ca^{2+} on the hydrolysis of [^{14}C]phosphatidylcholine is shown in Fig. 7. Both total hydrolysis and phospholipase A_1 activity



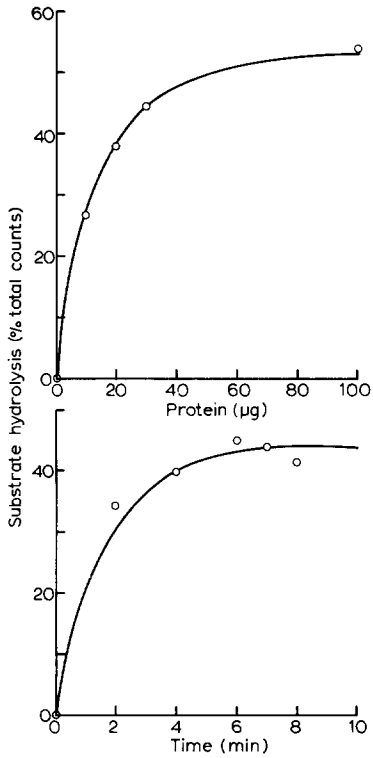


Fig. 5. Protein concentration and time curves for the hydrolysis of 2-([1-^{14}C]linoleyl)phosphatidylcholine at pH 6.0, substrate hydrolysis being measured from the sum of counts in lysophosphatidylcholine and free fatty acid after incubation.
Fig. 6. Protein concentration and time curves for the hydrolysis of [^{32}P]phosphatidylinositol at pH 6.0. Substrate hydrolysis measured as total hydrolysis from counts in upper phase following a water wash.

measured as counts in lysolecithin are shown. EDTA on the other hand had no significant effect on the hydrolysis. Similar inhibition both by Ca^{2+} and Mg^{2+} were shown for phosphatidylinositol substrate although the "two wash" procedure could not be used. Measurement of the contribution of lysophosphatidylinositol was made from thin-layer chromatograms.

A simple fractionation procedure located the phospholipase A_1 activity in both mixed pellet and microsomal fractions with higher activity in the latter. Heating the fractions at 70° for 5 min completely destroyed the enzymatic activity of both fractions.

DISCUSSION

The presence of phospholipase A activity in pancreas is well known. RIMON AND SHAPIRO[17] were able to detect activity in aged but not in fresh ox pancreas and this activity was stimulated by Ca^{2+}. A similar Ca^{2+}-stimulated phospholipase A_2 was found in human pancreas by MAGEE *et al.*[18], but these authors found no difference

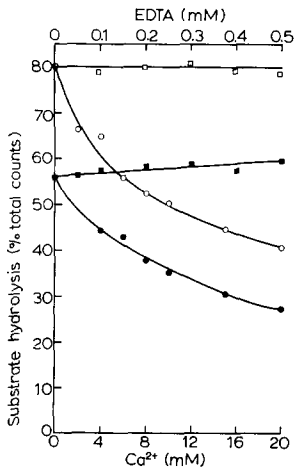


Fig. 7. Effect of Ca^{2+} and EDTA on the hydrolysis of 2-([1-^{14}C]linoleyl)phosphatidylcholine at pH 6.0 ○—○, effect of Ca^{2+} on total hydrolysis measured by decrease in counts in phosphatidylcholine; ●—●, effect of Ca^{2+} on phospholipase A_1 activity measured by increase in counts in lysophosphatidylcholine; □—□, effect of EDTA on total activity; ■—■, effect of EDTA on phospholipase A_1 activity.

between fresh and aged pancreas. This enzyme was heat stable and had an optimum at pH 9. VAN DEN BOSCH *et al.*[2] indicated the presence of both A_1 and A_2 activities in horse and rat pancreas using a doubly labelled phosphatidylcholine substrate. Their assay system contained 100 mg homogenate protein incubated at pH 7.4 for 2 h. The A_2 activity of both MAGEE *et al.*[18] and VAN DEN BOSCH *et al.*[2] was probably due to the enzyme isolated by DE HAAS *et al.*[19] which was a trypsin-activated, Ca^{2+}-stimulated, heat-stable phospholipase A_2 derived from a zymogen precursor[20].

Determination of phospholipase activity in guinea pig pancreas in the present work required a much shorter incubation time (10 min) and less than 100 μg protein to maintain zero-order conditions. A pH curve of phospholipase A activities showed two optima at pH 6 and pH 8.5 with both phosphatidylinositol and phosphatidylcholine as substrates. The results using 2-([1-^{14}C]linoleyl)phosphatidylcholine as substrate confirms the A_1 activity reported by VAN DEN BOSCH *et al.*[2]. It appears that phosphatidylinositol is the better substrate. The smaller peak of activity at pH 8.5 probably represents some phospholipase A_2 activity from the zymogen-derived enzyme.

It seems unlikely that the phospholipase A_1 activity detected at pH 6.0 was due to lipase, however this might well account for the activity at pH 8.5. DE HAAS *et al.*[21] and BROCKERHOFF[22] have shown that highly purified pancreatic lipase shows hydrolytic activity towards the 1-acyl group of phospholipids although the activity towards phospholipids is much less than towards triglyceride. Using labelled triglyceride (glyceryl [1-^{14}C]tripalmitate, final concentration 130 μM) we were unable to show appreciable activity under the experimental conditions routinely used.

The appearance of glycerylphosphorylinositol as the main product at pH 6.5 when phosphatidylinositol was substrate shows the presence of an active lysophospholipase. This result contrasts with that of DAWSON[23], who showed the main

hydrolysis product with an extract of ox pancreas to be inositol monophosphate resulting from phospholipase C activity. This enzymatic activity also had an optimum pH of 6 and was markedly stimulated by Ca^{2+}. We found some inositol phosphate as a product of the reaction at pH 6 but this might be derived from a phosphodiesterase acting on glycerylphosphorylinositol and not from phospholipase C. Thus if any phospholipase C activity is present it is very low due to the active phospholipase A which competes for substrate.

The enzyme described in this paper also differs from previous reports in not requiring Ca^{2+}. It had previously been reported that trypsin inhibitor had no effect on the activity[1]. In fact Ca^{2+} had a marked inhibitory effect on the enzyme whilst EDTA had no significant effect. Recently COOPER AND WEBSTER[24] have described phospholipase A_1 and A_2 activities in human and rat brain. Neither enzyme required Ca^{2+} and both were heat labile. However, the pH optimum of the A_1 enzyme was more acidic (pH 4.2–4.5) than for the A_1 enzyme of pancreas.

The subcellular distribution of the activity between the mitochondrial and microsomal fractions resembles the distribution of the enzyme in intestinal mucosa described by EPSTEIN AND SHAPIRO[25] which had a pH optimum of 6.5. This is suggestive of a lysosomal origin (*cf.* SMITH AND WINKLER[26]) but we were unable to detect appreciable activity of enzymes typical of lysosomes in the pancreas of various animals. Although FREXINOS *et al.*[27] claimed to have detected lysosome-like structures in electron micrographs of pancreas, we found that the activities of β-galactosidase, β-glucuronidase and acid phosphatase were either absent or very low in pancreas of cat, ox, guinea pig and rat. None of these enzymes showed any latency effect generally associated with lysosomal enzymes.

Phospholipase A_1 activity has been described in many tissues (*e.g.* rat liver[28], calf spleen[29] and rat lung[30], post-heparin human and rat plasma[31], and calf and rat brain[32]) and generally these enzymes have acidic pH optima (brain and spleen enzymes have optimum pH of 4), possibly being derived from lysosomes. ILLINGWORTH AND GLOVER[33] have demonstrated both phospholipase A_1 and A_2 at pH 7.4 in fresh human cerebrospinal fluid. More detailed fractionation studies of rat liver by SCHERPOF *et al.*[34], WAITE AND VAN DEENEN[28] and WAITE[35] have localized the phospholipase A activities in mitochondrial (A_2) and microsomal (A_1) subfractions. The use of phosphatidylinositol as a substrate for phospholipase preparations has generally been restricted to venom phospholipases[14,36] although ATHERTON AND HAWTHORNE[37] and DAWSON[23] used it as substrate for phospholipase C of intestinal mucosa and pancreas, respectively.

The rôle of the phospholipase A_1 described in the present paper is as yet unknown. It is interesting to note however that phosphatidylinositol is a good substrate and is rapidly hydrolyzed, in view of the fact that this phospholipid is rapidly turned over during stimulation of the pancreas with acetylcholine or pancreozymin[1], the so-called "phospholipid effect". This poses the question as to whether phosphatidylinositol is the natural substrate for the enzyme and in particular the phosphatidylinositol of the plasma membrane, where changes occur during fusion of the zymogen granules with the membrane.

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