BBA 66063

PURIFICATION AND PROPERTIES OF *CLOSTRIDIUM WELCHII* PHOSPHOLIPASE C

BRUCE A. DINER
The Rockefeller University, New York, N.Y. 10021 (U.S.A.)

(Received September 24th, 1969)

SUMMARY

Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Clostridium welchii* has been purified 32-fold over a commercial preparation. The purification involves $(NH_4)_2SO_4$ fractionation, adsorption and elution from calcium phosphate gel, DEAE-cellulose chromatography, and gel filtration through Sephadex G-100. From the appearance of acrylamide gel electrophoresis, the resulting enzyme is thought to be approx. 60–70% pure.

A new method of assay is described, which permits rapid and quantitative analysis of enzyme activity. Some kinetic data is also presented.

INTRODUCTION

Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) has gained increasing attention in recent years as a potential tool for the study of membranes. Its importance stems from the marked preference for L-α-lecithin as substrate, yielding D-1,2-diglyceride and phosphorylcholine, and from the fact that the enzyme can hydrolyse lecithin molecules *in situ* in a membrane. For the optimal application of the enzyme in this manner, a high degree of purity would be desirable. This paper describes a method of purification and some of the kinetic properties of *Clostridium welchii* phospholipase C.

EXPERIMENTAL PROCEDURE

Synthesis of substrate

Substrate, L-α-lecithin, was prepared by biosynthesis and by chemical synthesis. A choline-dependant mutant of *Neurospora crassa* (34486 R 10, generously provided by Dr. David Luck) was grown in minimal medium[1], supplemented with [*Me*-^{3}H]-choline. After 18.5 h, the mycelia were harvested, suspended in a solution of chloroform–methanol (2:1, v/v) and ground with washed sand in a chilled mortar and pestle. The homogenate was worked up for the isolation of total lipids[2]. ^{3}H-labelled,

lecithin was fractionated using silicic acid (Unisil, fine mesh, Clarkson Chem. Co.)[3,4] and was found to be homogeneous on Eastman Kodak silica gel plates. The plates were activated by heating for 1 h at 110°, and lipid was then chromatographed with chloroform–methanol–water (65:25:4, by vol.). The lipid was located by exposure to I_2 vapor and the radioactivity of segments of the chromatogram was determined in a scintillation counter. Localization of lecithin and radioactivity were found to be identical. Specific activity was 4.05 mμC/mμmole lecithin.

L-α-Dioleoyl lecithin was prepared by direct organic synthesis. 10 g of crude egg lecithin (Sigma Chemical Co.) were hydrolysed for 26 h in refluxing 50% ethanol, containing 0.184 M $HgCl_2$. The resulting glycerylphosphorylcholine was isolated as the $CdCl_2$ salt[5]. This procedure was known to give rise to a slightly racemic product, a problem of little import in that the stereoisomers were resolved later in the synthesis.

11 g of oleic acid (Mann Research Laboratories Inc.) were reacted with 4.25 ml $SOCl_2$ and the resulting oleoyl chloride was distilled at 0.2 mm Hg (b.p. 140°) using a fine capillary N_2 bleed[6]. The yield was about 9 g.

Dioleoyl lecithin was prepared by reacting 1.1 g of the $CdCl_2$ complex of glycerylphosphorylcholine with 6.0 g of oleoyl chloride, in the presence of pyridine[7]. The product, dioleoyl lecithin, was purified by silicic acid column and thin-layer chromatography. The latter involved batch purification on unactivated thick preparative plates of Silica Gel H (Brinckmann Instriuments Inc.) using chloroform–methanol–water (26:10:0.1, by vol.) as the solvent system. On each plate, the area containing lecithin was scraped off and placed directly onto a column containing a mixture of silicic acid (Unisil, fine mesh) and "Hyflo Super Cel" (2:1, w/w), previously equilibrated with chloroform. The last traces of oleic acid were removed by washing the column with chloroform. The dioleoyl lecithin (700 mg final product) was eluted with chloroform–methanol (3:2, v/v).

The purified lecithin was dissolved in 70 ml of ether–ethanol (98:2, v/v) and incubated at room temperature in the presence of 10 ml aqueous 0.1 M Tris–HCl (pH 7.3) and 5.0 mM $CaCl_2$, containing 100 μg previously purified phospholipase C (ref. 8). When the reaction ceased at about 50% degradation, the ether phase, containing diolein and undegraded lecithin, was evaporated *in vacuo*. The residue was dissolved in benzene and placed on a column (2 cm × 30 cm) containing Unisil–Hyflo Super Cel (2:1, w/w), equilibrated with benzene. The column was washed with several column volumes of benzene and elution with benzene–chloroform (85:15, v/v) gave 250 mg of D-1,2-diolein. The purpose of the enzymatic digestion was to select for stereospecific, *i.e.* degradable, L-α-dioleoyl lecithin. This step assured that the final labelled product would be of the L-form.

^{3}H-labelled L-α-dioleoyl lecithin was prepared from the D-1,2-diolein, phosphorusoxychloride, and [*Me*-^{3}H]choline (New England Nuclear, specific activity 50 mC/mmole) according to the method of BAER AND KINDLER[9]. Sufficient lecithin (4 mg, specific activity 49.6 mC/mmole) was obtained to permit enzyme purification and some kinetic analysis.

The products of the previous reactions were judged pure on the basis of extensive thin-layer chromatography and infrared spectroscopy. Phosphate analyses were performed by the method of AMES[10].

Assay of phospholipase C

A total assay volume of 100 μl contained: 40 μl 0.1 M Tris–HCl (pH 7.3); 40 μl 12.5 mM $CaCl_2$; 10 μl lecithin in abs. ethanol (8.80 mμmoles Neurospora lecithin/10 μl or 10.2 mμmoles dioleoyl lecithin/10 μl; 10 μl enzyme diluted in either 1 mg bovine serum albumin/ml 0.01 M Tris–HCl (pH 7.3) or 25% glycerol (v/v) in 0.01 M Tris–HCl (pH 7.3).

There was little difference in activity between bovine serum albumin- and glycerol-supplemented assays. Following preincubation for 45 sec at 37°, the assay was begun by the addition of 10 μl of diluted enzyme to the assay mix. After 15-min incubation at 37°, the reaction was stopped by the addition of 3 μl of 0.2 M Na_2EDTA. The incubation tubes were immediately chilled in an ice bucket and 1.0 ml of chloroform–methanol (2:1, v/v) was added, followed by 200 μl of 0.05 M KCl. The tubes were thoroughly swirled on a Vortex mixer and centrifuged at 0° for 5 min at 1085 $\times$ *g*. 250-μl aliquots of the upper phase (44.9% of a total upper phase volume of 557 μl) were pipetted into glass scintillation vials and dried thoroughly under an infrared lamp. After cooling, 1.0 ml methanol and 15 ml toluene–"Liquifluor" (New England Nuclear) were added and the vials swirled and counted in a scintillation counter.

An unit of enzyme activity is defined as the cleavage, from lecithin, of 1 μmole of phosphorylcholine/min, based on a 15-min assay.

Purification of Clostridium welchii phospholipase C

In the procedure that follows, synthetic dioleoyl lecithin was used as assay substrate and the Folin reagent used for determining protein. 0.9853 g of crude enzyme (Sigma Chemical Co., lot No. 38B-01500), containing 708 mg protein, were dissolved at 0° in 100 ml 0.01 M Tris–HCl (pH 7.6) containing 5.0 mM $CaCl_2$. The solution was diluted to 627 ml, with the same buffer, to yield a final protein concentration of 1.13 mg/ml.

123 g of solid $(NH_4)_2SO_4$ (enzyme grade) were added slowly, at 5°, with constant stirring, to a final concentration at 33% of saturation. After standing for 1–2 h, the precipitate was removed by centrifugation at 0° for 5 min at 10 400 $\times$ *g*. This precipitate contained less than 10% of the total enzyme activity. An additional 67.1 g of solid $(NH_4)_2SO_4$ were added to the supernatant to give a final concentration of 50% of saturation. The precipitate was permitted to form over a period of 12 h and was harvested as before. The supernatant was assayed, found to contain less than 1% of the total activity, and was discarded. The precipitate was redissolved in a minimal volume of 0.01 M Tris–HCl (pH 7.6) and dialysed for 18 h at 5° against the same buffer. The final volume of the dialysate was 35 ml and contained 72% of the initial enzyme activity.

33 ml of enzyme dialysate were mixed at 5° with 20 ml of calcium phosphate gel (13 ml packed gel volume)[11] and stirred for about 30 min. The gel was separated by centrifugation at 0° for 5 min at 4080 $\times$ *g*. The supernatant was poured off, and the gel washed and centrifuged 6 times with 30-ml volumes of 25 mM sodium phosphate buffer (pH 7.5). The loss of activity on washing was approx. 10–15%. Enzyme was eluted from the gel by three successive washes (20, 18, and 18 ml) and centrifugations with 1.0 M $(NH_4)_2SO_4$ (pH 7.5) at 5°. The elutions were pooled and dialysed twice, at 5°, against 0.01 M Tris–HCl (pH 7.6) containing 5.0 mM $CaCl_2$. Some loss in enzyme activity occurred during dialysis and was accompanied by the formation

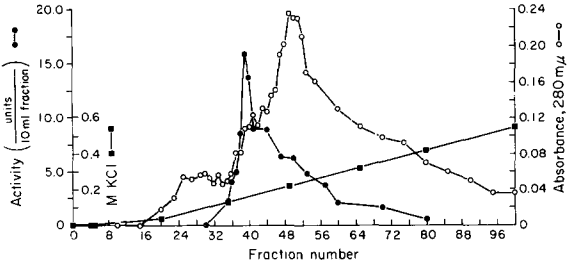
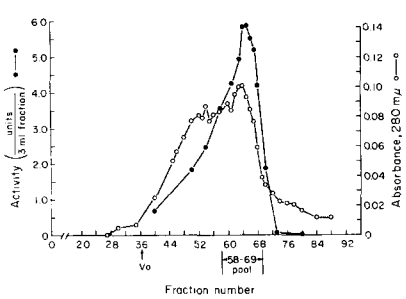


Fig. 1. DEAE-cellulose chromatography at 5° in 0.01 M Tris–HCl (pH 7.6) with a linear salt gradient extending from 0.0 to 0.6 M KCl. Flow rate and fraction volume were 60 ml/h and 10 ml, respectively.

of a small amount of whitish precipitate. 0.2 M $Na_4P_2O_7$ (pH 7.5) was occasionally used as eluant and demonstrated the same eluting properties as $(NH_4)_2SO_4$.

The dialysed enzyme was applied, at a flow rate of about 12 ml/h, to a DEAE-cellulose column (2.0 cm × 33 cm), previously equilibrated with 0.05 M Tris–HCl (pH 7.6) at 5°. The enzyme was eluted by a linear gradient, extending from 0.01 M Tris–HCl (pH 7.6) to 0.01 M Tris–HCl (pH 7.6) containing 0.6 M KCl, at a flow rate of 60 ml/h. 10-ml fractions were monitored for protein spectrophotometrically and assayed in the usual manner. The absorbance at 280 mμ and enzyme activity profiles are plotted in Fig. 1. Fractions 36–45 (100 ml) were pooled and placed in dialysis tubing. The pool was concentrated against dry sucrose and dialysed against 0.05 M Tris–HCl (pH 7.6) containing 5.0 mM $CaCl_2$, giving a final volume of 2.75 ml.

The concentrated enzyme was placed on a column of Sephadex G-100 (2.0 cm × 89 cm), previously equilibrated with 0.05 M Tris–HCl (pH 7.6) containing 5.0 mM $CaCl_2$ and 0.1 M KCl. Filtration proceeded with the same buffer at a flow rate of 7.2 ml/h. Fraction and exclusion volumes were 3 and 110 ml, respectively. A plot of absorbance at 280 mμ and enzyme activity is shown in Fig. 2. Fractions 58–69 were pooled, placed in dialysis tubing, and concentrated against dry Sephadex G-200.



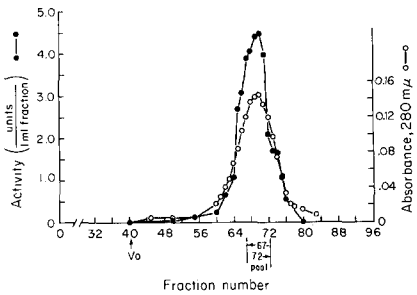


Fig. 2. First Sephadex G-100 filtration at 5° in 0.05 M Tris–HCl (pH 7.6) containing 5 mM $CaCl_2$ and 0.1 M KCl. 3-ml fractions were collected at a flow rate of 7.2 ml/h. The void volume, V_0, was about 110 ml.

Fig. 3. Second Sephadex G-100 filtration at 5° in 0.05 M Tris–HCl (pH 7.6) containing 5 mM $CaCl_2$ and 0.1 M KCl. 1-ml fractions were collected at a flow rate of 2.6 ml/h. The void volume, V_0, was about 40 ml.

The concentrate, less than 1 ml, was placed on a second Sephadex G-100 column (1.2 cm × 91 cm), previously equilibrated with 0.05 M Tris–HCl (pH 7.6) containing 5.0 mM $CaCl_2$ and 0.1 M KCl. Flow rate and fraction volume were 2.6 ml/h and 1.0 ml, respectively. The exclusion volume was 40 ml. The 280-mμ absorbance and enzyme activity profiles appear in Fig. 3. Fractions 67–72, containing a total of 0.317 mg of protein and 22.7 units of enzyme activity, were placed in dialysis tubing and concentrated against dry Sephadex G-200. The concentrate was dialysed against 0.05 M Tris–HCl (pH 7.6) containing 5.0 mM $CaCl_2$, giving a final volume of 300 μl. This final concentrate represented 0.188 mg of protein and 13.6 units of enzyme activity. 150 μl of glycerol were added to give a solution 33% (v/v) in glycerol. This final fraction was stored at −15° and was found to be stable, under these conditions, for longer than 6 months.

The entire purification procedure was repeated three times giving similar results. The data, from the purification described above, appears, in summary, in Table I.

Enzyme was electrophoresed on 10% acrylamide gels at pH 8.9 in 0.2 M Tris–HCl. A major band, comprising 60–70% of the total protein, was observed, in addition to a number of minor bands. While attempts to recover activity from the gel were unsuccessful, the similarity of the activity and 280-mμ absorbance profiles of Sephadex G-100 filtration suggest that the major protein component is the enzyme.

TABLE I

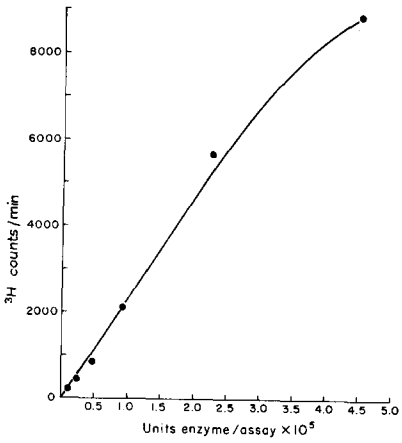
PURIFICATION OF *Cl. welchii* PHOSPHOLIPASE C

Enzyme was diluted with either 1 mg bovine serum albumin/ml or 25% glycerol and assayed in the usual manner. A unit of enzyme activity is equivalent to the release of 1 μmole of phosphorylcholine/min from lecithin, based on a 15-min assay. The specific activity is expressed as units of enzyme activity/mg of protein.

Step	*Enzyme units*	*Protein (mg)*	*Specific activity*	*% Recovery*
1 Start	1610	708	2.28	100
2 $(NH_4)_2SO_4$	1160	413	2.82	72.3
3 Calcium phosphate gel (before dialysis)	525	—	—	32.5
3a Calcium phosphate gel (after dialysis)	435	37.5	11.6	26.9
4 DEAE pool	132	6.25	21.0	8.15
4a DEAE pool (after dialysis and concn.)	118	4.48	26.2	7.27
5 1st Sephadex pool	63.0	1.16	54.3	3.39
6 2nd Sephadex pool	22.7	0.317	71.5	1.4
6a 2nd Sephadex pool (after dialysis and concn.)	13.6	0.188	72.3	0.84

RESULTS

The rate of degradation of lecithin, as a function of enzyme concentration, is plotted in Fig. 4. The hydrolysis of lecithin is a linear function of enzyme concentration up to a level of at least $2.27 \cdot 10^{-5}$ units per assay.



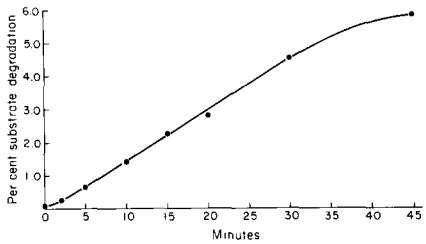


Fig. 4. Enzyme variation kinetics demonstrate linearity up to a concentration of at least $2.27 \cdot 10^{-5}$ units/100-μl assay. Counts/min are tritium in [*Me*-^{3}H]phosphorylcholine.

Fig. 5. Time-course of substrate degradation at 37°. 70 μl of 1 mg bovine serum albumin/ml in 0.01 M Tris–HCl (pH 7.3), containing $1.6 \cdot 10^{-4}$ units of enzyme, were mixed at zero time with a 630-μl solution containing: 280 μl 12.5 mM $CaCl_2$, 280 μl 0.1 M Tris–HCl (pH 7.3), and 70 μl dioleoyl lecithin (71.4 mμmoles/70 μl ethanol). At the indicated time points, 50-μl aliquots (one-half the usual 100 μl) were removed and mixed with 3 μl 0.2 M Na_2EDTA. The remainder of the workup was correspondingly scaled down by one-half but otherwise identical to the usual 100-μl assay procedure. The routine assay time period of 15 min is observed to be well within the linear range with a total substrate degradation of only 2.26%.

The time-course of enzyme action is given in Fig. 5. The 15-min incubation, used for routine assay, is well within the linear range, and corresponds to a substrate degradation of only 2.26%.

The Ca^{2+}-dependence for enzyme activity is shown in Fig. 6, which is in accord with the observations of others[12–15]. Using either mixed lecithin substrate (from Neurospora) or L-α-dioleoyl lecithin, an optimum Ca^{2+} concentration is obtained at approx. 5.0 mM. A slight, but reproducible, decrease in rate is observed at higher Ca^{2+} concentrations, amounting to not more than 10% of optimal activity.

A double reciprocal plot, Fig. 7, of $1/v$ *vs.* $1/[Ca^{2+}]$ gives a linear curve, suggesting first order participation of Ca^{2+}. Further consideration of Fig. 6 indicates that the $[Ca^{2+}]$ at $v_{max}/2$ is 0.5 mM, a value for a possible dissociation constant of Ca^{2+} with the enzyme. The argument for interaction of Ca^{2+} with enzyme is furthered by a study of heat denaturation, in which Ca^{2+} produces a marked stabilization of enzyme activity. Enzyme was diluted 1:5000 in 0.01 M Tris–HCl (pH 7.3) containing 1 mg bovine serum albumin/ml and 5.0 mM $CaCl_2$. The control was prepared in an identical manner except that it was deprived of Ca^{2+}. The enzyme solutions were maintained at 45° for 15 min, at which time assays were performed. Enzyme supplemented with Ca^{2+} retained 91% of the initial activity while deprived enzyme retained only 42%. After an additional 90-min at 45° the former had still retained 65% of the starting activity while the latter could demonstrate only 13.5%.

The variation of rate as a function of substrate concentration, for dioleoyl lecithin, is plotted in Fig. 8. Michaelis–Menten kinetics are demonstrated and satu-

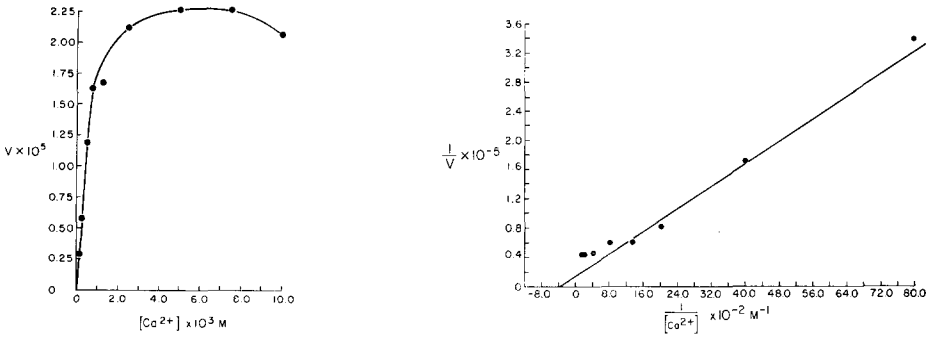


Fig. 6. Ca^{2+} activation kinetics showing maximal stimulation at 5 mM $CaCl_2$. 100-μl assays were conducted in the usual manner with a constant enzyme concentration of $2.27 \cdot 10^{-5}$ enzyme units per assay and Ca^{2+} concentration varied as indicated. Substrate was maintained at a constant concentration of 10.2 mμmoles per assay. v is the rate expressed in μmoles phosphorylcholine released, from lecithin, per min, based on a 15-min assay. The points plotted here are the average of a kinetic experiment run in triplicate.

Fig. 7. Double reciprocal plot of $1/v$ *vs.* $1/[Ca^{2+}]$. The data is that of Fig. 6, but is plotted here to indicate linearity.

ration occurs at about 0.1 mM. A double reciprocal plot, Fig. 9, indicates a K_m of approx. 70 μM.

For Neurospora lecithin, saturation occurs at a concentration of 88 μM. Substrate variation kinetics differ slightly from Michaelis–Menten kinetics in that they show some biphasic character. For this substrate the K_m appears to be somewhat lower, about 40 μM.

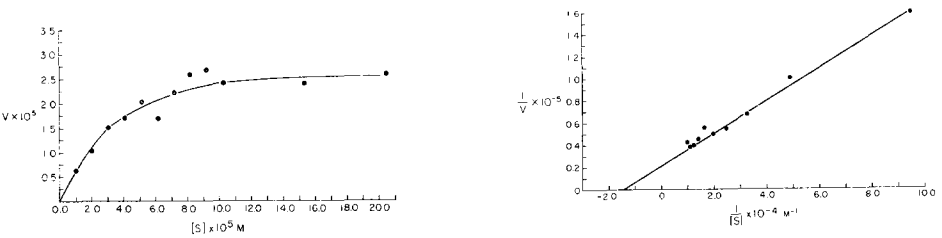


Fig. 8. Substrate variation kinetics with enzyme and Ca^{2+} concentrations constant at $2.27 \cdot 10^{-5}$ units/100 μl and 5 mM, respectively. v is the rate expressed as μmoles phosphorylcholine released, from lecithin, per min, based on a 15-min assay. Michaelis–Menten kinetics are observed. The points plotted here are the average of a kinetic experiment run in triplicate.

Fig. 9. Double reciprocal plot of $1/v$ *vs.* $1/[S]$. The data is that of Fig. 8 and serves to indicate linearity and intercept.

DISCUSSION

This paper has presented a new assay method and a new and more extensive purification of *Cl. welchii* phospholipase C.

The assay procedure is a direct and quantitative measure of the activity of the enzyme. Previous assay methods have included manometric[15], titrimetric[8], and water-

soluble phosphate determination[12], not to speak of mouse lethal dose and egg yolk turbidity assay. The present method combines a simple organic extraction with the sensitivity and accuracy of isotopic methods, permitting activity determinations two orders of magnitude more sensitive than those mentioned above.

The enzyme purification procedure presented here, while approx. 1% in overall yield, succeeds in purifying phospholipase C further than any previous method[12,14,16].* The most extensive procedure heretofore appears to have been that of BANGHAM AND DAWSON[14], but their lack of quantitative data precludes any comparison. Furthermore their $A_{280\ m\mu}/A_{260\ m\mu}$, indicating 5–10% contaminating nucleic acid, compares unfavorably to the $A_{280\ m\mu}/A_{260\ m\mu} = 1.84$ obtained here.

Purification techniques used previously have included: $(NH_4)_2SO_4$ (ref. 12), pH (ref. 14), and protamine precipitations[16], density-gradient electrophoresis[14], and Sephadex G-100 gel filtration[17]. The present purification has added calcium phosphate gel adsorption and elution and DEAE-cellulose chromatography to the list. The recent technique of isoelectric focusing was attempted here, but without success.

On considering the kinetic data, several points may made concerning the role of Ca^{2+} in the behavior of phospholipase C. Ca^{2+} kinetics, particularly the linearity of the double reciprocal plot, strongly suggest a first order participation of this ion. Thermal denaturation experiments show a considerable stabilization of enzyme by Ca^{2+}. While Ca^{2+} is undoubtedly associated with the lipid micelle, as BANGHAM AND DAWSON[14] have suggested, the present data suggests, in addition, a direct participation of this ion in an enzyme mechanism. It argues too, for a direct binding of Ca^{2+} to the enzyme. If as BANGHAM AND DAWSON[14] suggest, Ca^{2+} were to act solely as a source of positive charge with which to coat the surface of the micelle, then it might be argued that Mg^{2+}, with an equivalent affinity for distearoyl lecithin monolayers as Ca^{2+}, and Al^{3+} and Cu^{2+} with greater affinity[18], would be either equally or more active than Ca^{2+} in stimulating phospholipase C activity. That Mg^{2+} is less active and Al^{3+} and Cu^{2+} inhibitory[15] makes the ion–substrate argument alone unlikely and suggests further the possibity of a steric effect in Ca^{2+}–enzyme binding.

As for substrate variation kinetics, the K_m values obtained here are considerably lower than those obtained by HANAHAN AND VERCAMER[8], CHU[13], and ZAMECNIK *et al.*[15], who report values of 3.6–6.8 mM. The rather large discrepancy probably stems from a number of factors. The present system, 0.1 mM at lecithin substrate saturation, could readily contain substrate in a different physical state of aggregation from the above mentioned systems, which contain substrate greater than two orders of magnitude more concentrated at saturation. Furthermore, the micellar phase equilibria of lecithin are undoubtedly affected by the polarity of the medium in which the assay is performed. The present system is not a totally aqueous one as in the case of CHU[13], and ZAMECNIK *et al.*[15], nor predominately non-polar as in the method of HANAHAN AND VERCAMER[8]. In light of this argument it is curious that there isn't greater disagreement between the kinetic data of the totally aqueous systems and that of the organic system.

A further difference is the considerable purification of the enzyme under consideration here, which may have been purified free of competitive inhibitors responsible for raising the K_m.

* See NOTE ADDED IN PROOF, p. 522.

The only comment to be made about the slight peculiarity of the Neurospora substrate variation kinetics is that for the conditions under which the present assays were performed, kinetic data may have been complicated by the mixture of lecithins present.

It is apparent that for this enzyme, kinetic experiments are unfortunately complicated by the micellar nature of the substrate. It seems likely then, that additional characterization of substrate–substrate and substrate–enzyme interactions will probably come through further investigation into phase equilibria of lecithins in dilute aqueous medium, rather than through kinetic analysis. It is further hoped that eventual purification of the enzyme to homogeneity, followed by amino acid analysis and X-ray crystallographic data, will provide the ultimate insight into the question of substrate–enzyme and Ca^{2+}–enzyme interactions involved in phospholipase C.

NOTE ADDED IN PROOF (Received January 28th, 1970)

It has come to the attention of the author that in a paper by MACCHIA AND PASTAN[19] describing the effect of phospholipase C on the response of thyroid tissue slices to thyroid stimulating hormone, the enzyme was purified to a higher specific activity than was reported here. The absence of any additional physical data precludes making any further comparison between the two procedures.

ACKNOWLEDGMENTS

This investigation was supported in part by a grant (GM 10717-06) from the National Institutes of Health, U.S. Public Health Service. Thanks are due Dr. Edward Reich whose encouragement contributed to the completion of this work.

REFERENCES

1 H. J. VOGEL, *Microbial Genet. Bull.*, 13 (1956) 93.
2 J. FOLCH, M. LEES AND G. H. SLOANE STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
3 M. O. HALL AND J. F. NYC, *J. Lipid Res.*, 2 (1961) 321.
4 D. J. HANAHAN, V. C. DITTMER AND E. J. WARASHINA, *J. Biol. Chem.*, 228 (1957) 685.
5 N. H. TATTRIE AND C. S. MCARTHUR, *Can. J. Biochem. Physiol.*, 33 (1955) 761.
6 H. E. FIERZ-DAVID AND N. KUSTER, *Helv. Chim. Acta*, 22 (1939) 82.
7 E. BAER AND D. BUCHNEA, *Can. J. Biochem. Physiol.*, 37 (1959) 953.
8 D. J. HANAHAN AND R. VERCAMER, *J. Am. Chem. Soc.*, 76 (1954) 1804.
9 E. BAER AND A. KINDLER, *Biochemistry*, 1 (1962) 518.
10 B. N. AMES, *Methods in Enzymology*, 8 (1966) 115.
11 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 49 (1951) 88.
12 M. G. MACFARLANE AND B. C. J. G. KNIGHT, *Biochem. J.*, 35 (1941) 884.
13 H. P. CHU, *J. Gen. Microbiol.*, 3 (1949) 255.
14 A. D. BANGHAM AND R. M. C. DAWSON, *Biochim. Biophys. Acta*, 59 (1962) 103.
15 P. C. ZAMECNIK, L. E. BREWSTER AND F. LIPMANN, *J. Exptl. Med.*, 85 (1947) 381.
16 W. E. VAN HEYNINGEN AND E. BIDWELL, *Biochem. J.*, 42 (1948) 130.
17 H. IKEZAWA, A. YAMAMOTO AND R. MURATA, *J. Biochem.*, 56 (1964) 480.
18 P. J. ANDERSON AND B. A. PETHICA, in G. J. POPJÀK AND E. LEBRETON, *Biochemical Problems of Lipids*, Interscience, New York, 1956, p. 24.
19 V. MACCHIA AND I. PASTAN, *J. Biol. Chem.*, 242 (1967) 1864.