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STUDIES ON PHOSPHOLIPASE A AND ITS ZYMOGEN FROM PORCINE PANCREAS

III. ACTION OF THE ENZYME ON SHORT-CHAIN LECITHINS

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SUMMARY

1. Short-chain lecithins (with C_6, C_7, and C_8 fatty acid esters) have been used to study kinetically the enzymatic hydrolysis by pancreatic phospholipase A (EC 3.1.1.4) in aqueous systems, without the addition of emulsifiers.

2. Although phospholipase A is able to attack these substrates in molecularly dispersed form, micellar solutions are hydrolyzed at a much higher rate.

3. Of the three substrates examined, dioctanoyllecithin appeared to be the best substrate. Differences in maximal velocities might be interpreted in terms of interfacial area per molecule.

4. Ca^{2+} is specifically required for activity of pancreatic phospholipase A. The kinetic results are consistent with a random mechanism in which the metal ion combines with the enzyme independently of the substrate. The substrate was found to combine with the enzyme independently of the metal ion concentration.

5. Kinetic parameters were determined with diheptanoyllecithin as a substrate over a pH range from 5 to 9. Maximal binding of enzyme with substrate was observed at pH $\leqslant$ 6. The affinity of the enzyme for Ca^{2+} decreased at pH values below 6.5.

6. With diheptanoyllecithin as substrate, maximal velocities at infinite substrate and Ca^{2+} concentrations showed an optimum at pH 5.75.

7. NaCl at high concentrations (up to 3.9 M) gave a 80-fold stimulation of the v_{max} (diheptanoyllecithin as substrate). The K_s value decreased slightly with increasing salt concentrations, while the $K_{Ca^{2+}}$ increased very strongly. The activating effect of salt is presumed to be caused by a change of the properties of the lipid–water interface.

INTRODUCTION

Phospholipase A (EC 3.1.1.4) catalyzes the specific hydrolysis of fatty acid ester bonds at the 2-position of 1,2-diacyl-*sn*-phosphoglycerides[1]. A pH optimum in

Abbreviation: CMC, critical micelle concentration.

alkaline solution is reported generally, as is a requirement of Ca^{2+} for activity. This wide-spread enzyme has been purified from various snake venoms[2-11], bee venom[12,13], and from pancreatic tissues[14-17]. Characteristic properties of the enzyme are its high heat stability, its low molecular weight, and a compact structure caused by a high number of disulfide bridges. In the pancreas of man[18], rat[19], and pig[15] the enzyme does not occur in the active form, but is secreted as an enzymatically inactive zymogen. Limited proteolysis by trypsin produces the active enzyme. Recently, the complete amino acid sequence and the position of the disulfide bridges has been reported for the porcine pancreatic phospholipase A and its zymogen[20,21]. SAMEJIMA *et al.*[22], reported the partial sequence of a snake venom phospholipase A *(Agkistrodon halys blomhoffii)*.

In general, phospholipase A is not able to hydrolyze aqueous dispersions of long-chain lecithins. The lecithins are insoluble in water, but they produce stable dispersions by forming large lamellar structures, called liposomes. Such systems consist of closed, concentric lipid bilayers, separated from each other by water layers. Dependent on their chemical structure (degree of unsaturation, chain length) the lecithin molecules are more or less closely packed.

Addition of bile salts break the tightly packed lamellar structure down to much smaller mixed micelles[23], and a rapid hydrolysis of the lecithin molecules by phospholipase A occurs[16]. Also the addition of diethylether[24] or acidic phospholipids[25], which might introduce more space between the phospholipid molecules, can transform the inert liposomes into more loosely packed structures which are susceptible to phospholipase A attack.

It seems of interest to investigate more closely how the action of the enzyme might be controlled by specific properties of the lipid–water interface, such as area per phospholipid molecule, charge effects, etc. Unfortunately, incubation systems containing organic solvents or bile salts are too complicated to be useful for kinetic analysis of the enzyme.

To overcome these problems, ROHOLT AND SCHLAMOWITZ[26] proposed already in 1961 the use of the water-soluble dihexanoyllecithin as substrate. They were able to show that crude phospholipase A from *Crotalus durissimus terrificus* attacks this substrate in molecularly dispersed aqueous solution without the addition of detergents.

The present study is an attempt to further characterize the purified pancreatic enzyme with several synthetic short-chain 3-*sn*-phosphatidylcholines as substrates. Dependent on fatty acid chain length and lipid concentration, these lecithins give either molecularly dispersed, micellar or lamellar structures in water, and it seemed of interest to investigate the kinetic parameters of the enzyme as a function of such different lipid–water interfaces, and in addition as a function of metal ions, ionic strength, and pH.

MATERIALS AND METHODS

Enzyme source

Porcine pancreatic phospholipase A_2* was obtained by the activation of the pure zymogen with trypsin as described previously[15].

* This enzyme will be denoted as phospholipase A, unless otherwise stated.

Substrates

3-*sn*-Phosphatidylcholines with two identical acylchains varying in chain length between pentanoic acid (C_5) and nonanoic acid (C_9)* were prepared as described by CUBERO ROBLES *et al.*[27,28].

Assays

Enzymic hydrolysis of the synthetic lecithins was followed by continuous titration of the liberated fatty acids at constant pH (between pH 5 and 10) at 40° with a Radiometer pH-stat equipment. The assay-system was standardized to 4 ml total volume and contained lecithin, $CaCl_2$ and NaCl in various amounts. Depending on the pH, 0.5 mM Tris or acetate buffer was used. Lyophilized enzyme, dissolved in distilled water, was added in aliquots of 10–100 μl. Titrations with 0.04 M NaOH were carried out under nitrogen. In the pH range used, non-enzymatic hydrolysis of substrate was negligible. Initial rates of hydrolysis were measured under conditions where a good proportionality between enzyme concentration and activity existed. Analysis of the reaction products showed only the formation of a lysolecithin and fatty acid.

At pH values below 5.0, the pH-stat technique cannot be used to measure enzyme activity, and the hydroxamate method as modified by AUGUSTYN AND ELLIOT[29] was applied. Specific activity of the enzyme is expressed as the number of μMoles of fatty acid released per min per mg of protein (dry weight).

Titration efficiency

Between pH 5.0 and 7.0 corrections have to be made for the titration efficiency of C_6–C_8 fatty acid. Fig. 1 shows the percentage of fatty acid which is titrated under the average assay conditions. This pattern is independent of the Ca^{2+} concentration. At pH 7.0 the fatty acids are virtually completely ionized.

Critical micelle concentration determinations

Determinations of the critical micelle concentrations (CMC) were carried out by measuring the spectral shift induced upon incorporation of Rhodamine 6G into micelles, as described by BECHER[30].

RESULTS AND DISCUSSION

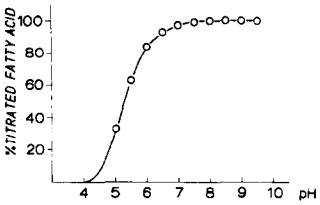
Activity of phospholipase A with short-chain lecithins as substrates

The activity of phospholipase A was examined with short-chain lecithins. These lecithins (diheptanoyllecithin and lower homologues) give optically clear solutions in water and no saturation point has been found up to concentrations of about 100 mg lecithin per ml. Micelle formation, however, occurs at low concentrations for dihexanoyllecithin and diheptanoyllecithin (13.8 and 2 mM, respectively**).

As shown in Fig. 2, micellar solutions of, for instance diheptanoyllecithin, are readily hydrolyzed by the pancreatic phospholipase A in the absence of detergents

* According to the tentative rules for lipid nomenclature (IUPAC) the preferred name for these compounds is: 1,2-diacyl-*sn*-glycero-3-phosphorylcholines. For the sake of simplicity these will be abbreviated to dipentanoyllecithin, *etc.*

** These values were confirmed by the light-scattering technique and by surface tension measurements.



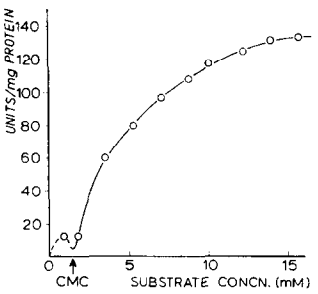


Fig. 1. Titration efficiency at various pH values for heptanoic and octanoic acid (5 and 10 μmoles). Titrations were carried out in a mixture of 10 mM of the corresponding lecithin, 0.1 M NaCl, 0.5 mM Tris, and 10 mM $CaCl_2$. This mixture is representative for an average assay. The amounts of alkali required to reach a certain pH value in the presence of fatty acid were corrected for a blank. The data are an average of three determinations.

Fig. 2. Michaelis curve of the hydrolysis of diheptanoyllecithin with phospholipase A. Assay conditions: 0.5 mM Tris, 0.1 M NaCl, 10 mM $CaCl_2$, pH 6.5, and varying concentrations of substrate.

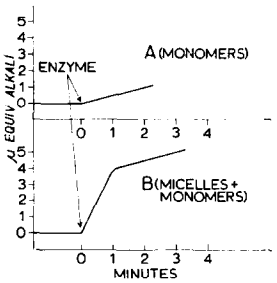
like sodium deoxycholate. Addition of this detergent had no influence on the rate of breakdown. In the micellar region (above the CMC) Michaelis curves are obtained which describe the progressive adsorption of the enzyme at the surface of the micelles. Finally, a maximal rate of hydrolysis is obtained when virtually all enzyme molecules are bound to the lipid–water interface.

Even at substrate concentrations below the CMC, where presumably mainly monomers are present, some enzymatic activity could be demonstrated*. The enzyme activity, however, does not increase continuously with increasing monomer concentration. It reaches a maximum and then falls off with higher amounts of substrate, finally giving a minimal activity at the CMC. In this monomeric region, however, a poor proportionality was found between activity and enzyme concentration. Hence evaluation of the kinetic constants v_{max} and K_m is rather difficult. Work is in progress to investigate by direct binding studies whether the enzyme is capable of binding monomeric lecithin molecules. In any case, the observed activity against "monomers" is low compared with the activity of phospholipase A against micelles. The preferential attack of substrate molecules present in micellar form is also shown in Fig. 3.

Enzyme activity is measured with two solutions of dihexanoyl-lecithin. Curve A represents the slow hydrolysis rate of this substrate at a concentration just below the CMC. Curve B with substrate concentration just above the CMC suggests that the small amount of substrate present in micellar form is hydrolyzed at a much higher rate. After about 1 min, however, most of the micelles are degraded and the remaining substrate is hydrolyzed by the enzyme at the same low rate as found in curve A. From these data it seems that phospholipase A has a pronounced preference to attack lecithin molecules present in certain organized structures. A similar behavior has been reported earlier for phospholipase A from *Crotalus adamanteus*[1].

ENTRESSANGLES AND DESNUELLE[31] also showed that the lipolytic enzyme, pancreatic lipase (EC 3.1.1.5)hydrolyzes short-chain triglycerides as emulsions and "micellar" solutions at a much higher rate than the corresponding monomeric solu-

* Also dipentanoyllecithin (with CMC greater than 60 mM) is very slowly hydrolyzed by the enzyme in the concentration range where monomers are supposed to be present.



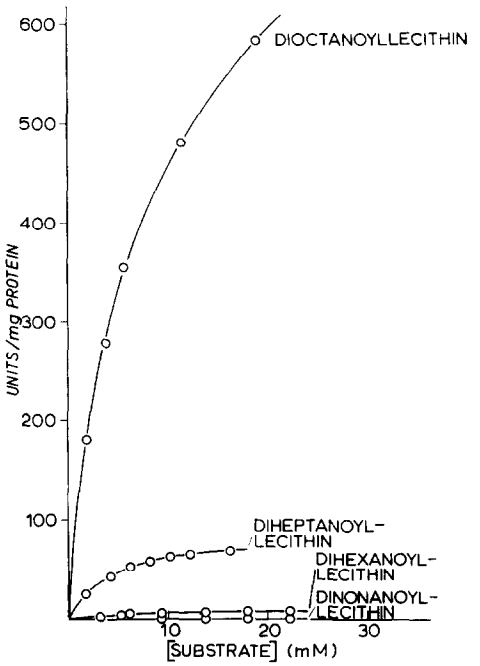


Fig. 3. Comparison of reaction velocities of the action of phospholipase A on monomeric lecithin molecules and on lecithin micelles. Assay conditions: pH 7.0, 0.1 M NaCl, 10 mM $CaCl_2$. A, titration curve for hydrolysis of 13.5 mM dihexanoyllecithin monomers (just below CMC); B, titration curve for hydrolysis of 14.5 mM dihexanoyllecithin (above the CMC, therefore a mixture of micelles and monomers). At the break point, after about 1 min, all micelles are hydrolyzed.

Fig. 4. Comparison of Michaelis curves of the hydrolysis of dinonanoyl-, dioctanoyl-, diheptanoyl-, and dihexanoyllecithin by phospholipase A. Assay conditions in all cases: pH 7.0, 0.1 M NaCl, 0.5 mM Tris, and 1 mM $CaCl_2$. Substrate concentrations are expressed in mmoles/l substrate molecules present as micelles or liposomes respectively.

tion. Their finding that the enzyme displays a similar v_{max} for the same substrate present in "micelles" and in emulsion droplets might indicate a closely related architecture of both interfaces, which determines the rate limiting step in the hydrolysis.

In order to investigate the influence of enzyme activity on a gradually changing lipid–water interface, a homologous series of short-chain lecithins was used as substrate. Light scattering experiments showed that the optically clear dihexanoyl- and diheptanoyl-lecithin form spherical micelles containing about 25 and 40–50 monomers, respectively*. The dioctanoyllecithin, however, gives a turbid solution in water, even after ultrasonic treatment. This system is not a stable dispersion, but readily separates into two layers, a very viscous lipid-rich lower phase and a clear supernatant containing the lecithin at a level of less than 0.3 mg/ml**. As judged from the high viscosity, this lecithin produces probably large disc-shaped or elongated cylindrical micelles in

* Experimental details on the determination of micellar molecular weights of ionic detergents by light scattering are described by HUISMAN[32]. These measurements were carried out under similar conditions of pH and ionic strength as used for the kinetic experiments.

** X-ray investigation of the coacervate containing about 5% lipid showed the absence of lamellar and hexagonal structure. (Personal communication from Miss A. TARDIEU, Laboratoire de Génétique Physiologique, C.N.R.S., Gif-sur-Yvette, France).

TABLE I

KINETIC PARAMETERS OF PANCREATIC PHOSPHOLIPASE A WITH SHORT-CHAIN LECITHINS AS SUBSTRATES

Assays were performed as described under methods. The assay conditions (pH 7.0, 1 mM $CaCl_2$, and 0.1 M NaCl) for the three substrates were arbitrarily chosen, but are representative for the comparison of the kinetic parameters. v_{max} and K_s* values were obtained from Lineweaver–Burk plots. v_{max} is expressed in μmoles/mg per min. K_s is expressed as concentration of substrate present in the form of micelles.

Substrate	v_{max} *(at* $[Ca^{2+}] = 1$ *mM)*	K_s *(mM)*
Dihexanoyllecithin	10	6.3
Diheptanoyllecithin	45	4.7
Dioctanoyllecithin	820	7.4

* K_s represents the dissociation constant of the enzyme–substrate complex as will be explained later.

water which are insoluble and in equilibrium with monomeric species. Dinonanoyllecithin is the first of the higher homologues of lecithin which form liposomes in water as could be demonstrated by phase-contrast microscopy*.

As shown in Fig. 4, large differences in enzyme activity exist even for these chemically very related substrates. As was anticipated, liposomes of dinonanoyllecithin are not degraded by the enzyme. In order to compare the enzyme action against the micelles of the lower homologues, the molar substrate concentrations plotted on the abscissa have been corrected for the amount of substrate present as monomers (CMC). The assumption is made that in these micelles all molecules are exposed to the solvent and in principle are capable of reacting with the enzyme. In this way the main difference with a true solution is the regular organization of the molecules in a lipid–water interface. No corrections were made for the low enzyme activity toward monomers.

By converting the Michaelis curves of Fig. 4 into Lineweaver–Burk plots[34], the kinetic parameters of the enzyme with the various substrates are obtained (see TABLE I). The K_s values** which are expressed in M substrate entirely present in the micellar state show roughly a constant value for the three substrates. However, since micellar size and hence the number of molecules per micelle and area per molecule change, features which have been neglected in our assumption, a direct comparison of the K_s values is not justified. TABLE I shows an 80-fold increase in v_{max} (at $[Ca^{2+}] = 10^{-3}$ M) going from dihexanoyl- to dioctanoyllecithin. Apparently the differences in architecture of the micellar structures of these related lecithins have a very large bearing on subsequent reaction steps during catalysis. If we assume a classical acylation–deacylation mechanism also for this special hydrolase, we are forced to conclude that at least the rate-limiting step must be strongly influenced by one (or more) of the parameters of the interface. An attractive hypothesis which was originally suggested by HUGHES[35] and by SHAH AND SCHULMAN[36] might be to consider the area per molecule of substrate in the interface (or the charge density per unit of surface) as one of the most important factors which govern the rate of hydrolysis. Calculations of the area per molecule for spherical micelles of dihexanoyl- and diheptanoyllecithin

* Observations were made under a light microscope fitted with crossed nicol prisms and a first-order red compensator[38].

** In the next section it is made plausible that this dissociation constant is indicative of the true equilibrium constant of the enzyme–substrate complex.

give approximate values of 125 Å² and 100 Å², respectively. Although the exact geometry of dioctanoyllecithin micelles is still unknown, it seems to be reasonable to attribute an area of approximately 90 Å² per molecule to this, still micellar, interface, taking into account the expected close packing of dinonanoyllecithin molecules in liposomes with an area per molecule of approximately 75–80 Å².

The slow hydrolysis by the enzyme of dihexanoyl- and diheptanoyllecithin micelles and the high susceptibility of the dioctanoyllecithin micelles together with the observed inertness of the dinonanoyllecithin liposomal structure might indicate that an area per lecithin molecule in the interface of about 90 Å² provides a highly favorable packing of the molecules for the enzyme. In this respect it should be remarked that the enzymatic degradation of dioctanoyllecithin spread as a monomolecular layer[45] shows a pronounced maximum at a pressure of 8 dynes/cm, which is equivalent to an interfacial area per molecule of approximately 90 Å². Also monolayer studies of COLACICCO AND RAPPORT[37] who investigated the breakdown of natural, long-chain lecithins by two different snake venom phospholipases A, demonstrated an optimal action of the enzymes at a surface pressure of 12 dynes/cm, which also corresponds with an area per molecule of about 90 Å².

Kinetic parameters of pancreatic phospholipase A with short-chain lecithins as substrate. Proposed mechanism

The pancreatic enzyme is completely inactive in the standard assay system if Ca^{2+} is omitted[14]. Fig. 5 shows that the enzyme absolutely requires Ca^{2+} at all pH values studied, suggesting a role of this metal ion in the fixation of phospholipase A to its substrates and/or in the reaction steps of the catalytic process itself. As will be shown in a subsequent paper, the requirement of the enzyme for Ca^{2+} is absolutely specific and no other common metal ion can substitute for it*. Ba^{2+} and Sr^{2+} are pure

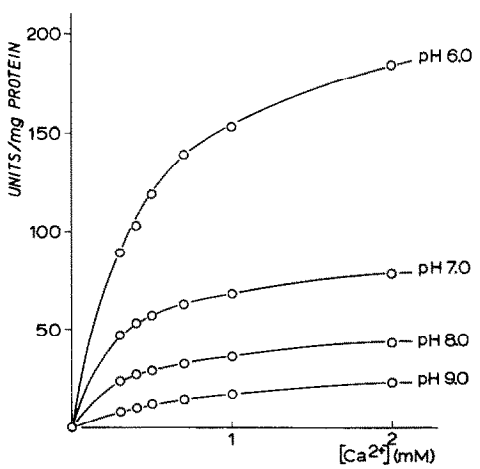


Fig. 5. Effect of pH on the requirement for Ca^{2+} in the hydrolysis of diheptanoyllecithin with phospholipase A. Assay conditions: 0.5 mM Tris, 0.1 M NaCl, 14 mM diheptanoyllecithin, and varying $CaCl_2$ concentrations. Measurements were performed at pH values as indicated.

* The purified enzyme contained a low amount of calcium as measured by titration with murexide[33]. This calcium was mainly introduced during the dialysis against distilled water. No indication for the presence of other metal ions was obtained.

competitive inhibitors for phospholipase A, in other words they are able to bind to the enzyme with a dissociation constant close to that of Ca^{2+}. However, the enzyme–metal–substrate complex formed did not lead to product formation.

Mg^{2+} is unable to bind to the enzyme and its presence has no influence on enzyme activity measured with Ca^{2+}.

What is the pathway leading to the formation of the active enzyme–metal–substrate complex? In order to investigate more closely the possible pathways, initial reaction rates of the enzyme with diheptanoyllecithin were measured as a function of varying substrate and Ca^{2+} concentrations and pH.

Fig. 6a shows at pH 7.0 the double reciprocal plots of enzyme activity *versus* substrate concentrations for various fixed concentrations of $CaCl_2$. The expermental points fit straight lines which gave a common intersection on the abscissa after extrapolation. This intersection was used to calculate the dissociation constant K_s of the enzyme–substrate complex. In Fig. 6b, at the same pH, double reciprocal plots of enzyme activity *versus* Ca^{2+} concentrations are given for various fixed substrate concentrations. Again straight lines were obtained with a common intersection on the X-axis which was used to calculate the dissociation-constant $K_{Ca^{2+}}$ of the enzyme–metal complex. Similar Lineweaver–Burk patterns with common intersections on the abscissa were obtained at various pH values between 5.0 and 9.0, indicating an identical mechanism for the formation of the active enzyme–metal–substrate complex in the pH range studied. ROHOLT AND SCHLAMOWITZ[26] discussed two ordered pathways being both in agreement with their inhibition experiments with Ba^{2+}. In the first pathway, the enzyme binds first reversibly with Ca^{2+}, and the enzyme–Ca^{2+} complex forms subsequently the active enzyme–metal–substrate complex:

$$E + Ca^{2+} \rightleftharpoons E \cdot Ca^{2+} \overset{S}{\rightleftharpoons} E \cdot Ca^{2+} \cdot S \qquad (1)$$

According to the second mechanism, there is first a formation of an enzyme–substrate complex which then reacts further with Ca^{2+} to form the enzyme–metal–substrate complex:

$$E + S \rightleftharpoons E \cdot S \overset{Ca^{2+}}{\rightleftharpoons} E \cdot Ca^{2+} \cdot S \qquad (2)$$

A third possibility is that not the free lecithin, but a Ca^{2+}–lecithin complex is the true substrate for the enzyme; but this was rejected by these authors as being not in agreement with their kinetic data. Our failure to demonstrate any binding of Ca^{2+} with the short-chain synthetic lecithins forced us to consider the last mechanism also as extremely improbable.

Direct binding studies in our laboratory, the result of which will be reported in a subsequent paper, are not in disagreement with the ordered mechanisms (1) and (2). In other words, the enzyme is indeed capable of binding Ca^{2+} in the absence of substrate, and the enzyme can form a complex with lecithins without the presence of Ca^{2+}. However, the kinetic results as shown in Figs. 6a and 6b do not support an ordered mechanism. According to pathway (1), one would expect a reaction velocity for infinite substrate concentration which is independent of the Ca^{2+} concentration. In other terms, Lineweaver–Burk plots as shown in Fig. 6a should give a common intersection on the ordinate, which is clearly not true in this case. The second pathway (2) is characterized by a reaction velocity which becomes independent of substra-

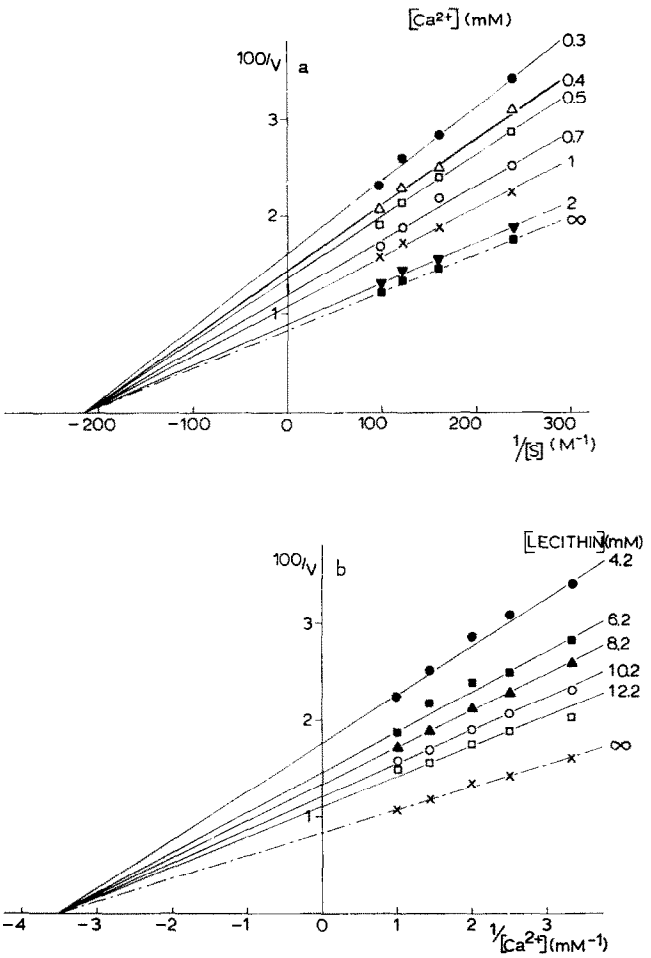
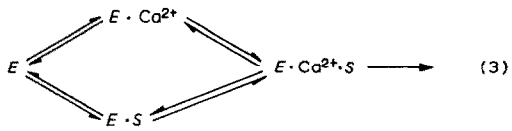


Fig. 6 (a) Lineweaver–Burk plots of hydrolysis of diheptanoyllecithin by phospholipase A at pH 7.0 with varying Ca^{2+} concentrations. Assay conditions: 0.5 mM Tris or acetate buffer, 0.1 M NaCl, varying substrate concentrations, and 5 different $CaCl_2$ concentrations as indicated. The dotted line for $[Ca^{2+}]$ = infinite was obtained from Fig. 6b by plotting the $1/v'_{max}$ values *versus* $1/[S]$. The intersection of the dotted line with the ordinate represents $1/v_{max}$ at infinite Ca^{2+} concentration. $[S]$ is expressed as moles/l of lecithin present in the form of micelles. Similar patterns were obtained for all pH values between 5 and 9. (b) Double reciprocal plots of reaction velocities and Ca^{2+} concentrations obtained from hydrolysis of varying substrate concentrations (diheptanoyllecithin) by phospholipase A. Assay conditions: the same as in (a). The dotted line for $[S]$ = infinite is obtained from (a) by plotting the $1/v_{max}$ values *versus* the reciprocal of the Ca^{2+} concentration. The intersection of the dotted line with the ordinate represents $1/v'_{max}$ at infinite substrate concentration and equals $1/v_{max}$ at infinite Ca^{2+} concentration (see a). Similar patterns were obtained for all pH values between pH 5 and pH 9.

te concentration at infinite Ca^{2+} concentration. Plots of this type (see Fig. 6b) should give again a common intersection on the ordinate which is apparently not the case. Figs. 6a and 6b do show a common intersection on the abscissa which can be expected for a random mechanism in which the metal ion acts by combining with the enzyme

independently of the substrate and the substrate combines with the enzyme independently of the Ca^{2+} concentration:



(3)

In this case, described by DIXON AND WEBB[39] as Case I, the common intersection on the abscissa (Fig. 6a) allows us to calculate the K_s value, the dissociation constant of the E–S complex, whereas $K_{Ca^{2+}}$, the dissociation constant of the enzyme–metal complex, directly follows from the intersection in Fig. 6b.

The pH dependence of the kinetic parameters K_s and $K_{Ca^{2+}}$ obtained from Figs. 6a and 6b at various pH values is shown in Fig. 7. Although the pH-stat assay technique did not allow us to measure enzyme affinity at pH values below 5.0, it seems that optimal binding between enzyme and substrate takes place at slightly acidic conditions. Direct binding experiments between enzyme and shortchain lecithins, which will be reported later, indicate a binding constant at pH 4.0 which is very close to the

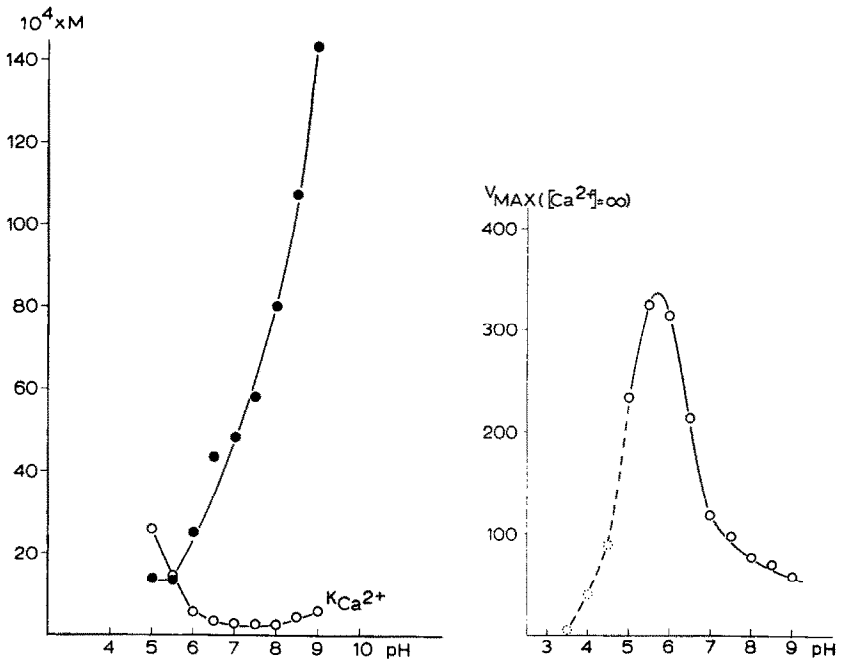


Fig. 7. Dependency of K_s and $K_{Ca^{2+}}$ on pH for hydrolysis of diheptanoyllecithin with phospholipase A. K_s and $K_{Ca^{2+}}$ values were obtained from Figs. 6a and 6b measured at pH values between 5 and 9. At all pH values common intersections with the abscissa were observed in both double reciprocal plots. K_s is expressed in moles/l of substrate in micellar form (K_s varies between 1.3 and 16 mM (●), and $K_{Ca^{2+}}$ between 2.6 and 0.25 mM (○).

Fig. 8. pH optimum of pancreatic phospholipase A hydrolysis of diheptanoyllecithin. Maximal velocities were obtained from Figs. 6a and 6b at infinite substrate and Ca^{2+} concentration. Assay conditions as described in Fig. 6a. The values below pH 5 were obtained by the hydroxamate method at a fixed high Ca^{2+} concentration.

values found at pH 5.0 and 5.5. Above pH 5.5 one observes a rapid decrease in binding efficiency. As regards the binding of Ca^{2+} by the enzyme, it is obvious that slightly alkaline conditions favor this association. At pH values below 6.0, a rapid decrease in affinity between metal and enzyme can be observed. Again a very similar pH dependence of the phospholipase A–Ca^{2+} binding was found by direct binding studies.

From the experiments described in Figs. 6a and 6b, which were carried out at nine different pH values between 5.0 and 9.0, maximal velocities for infinite Ca^{2+} and infinite substrate concentrations are obtained in the following way: the intersections on the ordinate in Fig. 6a represent the reciprocal values of maximal velocity for infinite substrate, but defined Ca^{2+} concentration. These values were plotted *versus* the reciprocal Ca^{2+} concentration (dotted line in Fig. 6b). The intersection with the ordinate then gives the reciprocal value for v_{max} at infinite substrate and Ca^{2+} concentration. The corresponding intersections with the ordinate in Fig. 6b can be transformed into the dotted line in Fig. 6a. The intersection of this line with the ordinate also gives the reciprocal value of v_{max} ([S] and $[Ca^{2+}]$ = infinite). Fig. 8 shows the pH dependence of the so-obtained v_{max} values. Below pH 5.0 enzymatic activities had to be measured by the hydroxamate method. Although various substrate concentrations were used, the assays were performed at only one high Ca^{2+} concentration. The obtained extrapolated v_{max} values are therefore dotted, but are indicative of a rapid decrease of the v_{max} at infinite Ca^{2+} concentration at these pH values. With these short-chain lecithins as substrate, phospholipase A has a pronounced pH optimum between pH 5.5 and 6.0. The same pH optimum of about pH 6 was also obtained by monolayer techniques with dioctanoyllecithin as substrate.

From this result one might speculate that for the decomposition of the metal–enzyme–substrate complex at least one amino acid side chain is important which should be in unprotonated form, with a pK value around 4.75 (presumably a carboxyl group).

The pH optimum of pancreatic phospholipase A *in vitro* is generally found to be around pH 8.0 It cannot be precluded, however, that under the conditions of these measurements an apparent pH optimum is found due to the fact that bile salts loose their emulsifying action at more acidic pH values.

Influence of high NaCl concentrations on the activity of phospholipase A with short-chain lecithins as substrate.

As was shown in Fig. 4, the enzyme activity against micellar interfaces of closely related lecithins is strongly dependent on the chain-length of the substrate. We supposed that small differences in the architecture of the lipid–water interfaces, for instance the area per molecule, are responsible for these large effects on enzyme activity. That the relatively low activity of the enzyme *versus* dihexanoyl- and diheptanoyllecithin compared with the dioctanoyl derivative is probably caused by physicochemical differences of the interfaces, is supported by the finding that high ionic strength enhanced reaction velocities.

As shown in Fig. 9, the addition of large amounts of NaCl to aqueous solutions of diheptanoyllecithin not only lowers considerably the CMC, but also increases dramatically the activity of the enzyme at the same time. A similar activating effect was noted with dihexanoyl- and dioctanoyllecithin as substrate. Maximal velocities at infinite substrate concentration ($[Ca^{2+}]$ = 1 mM) were obtained from Lineweaver–

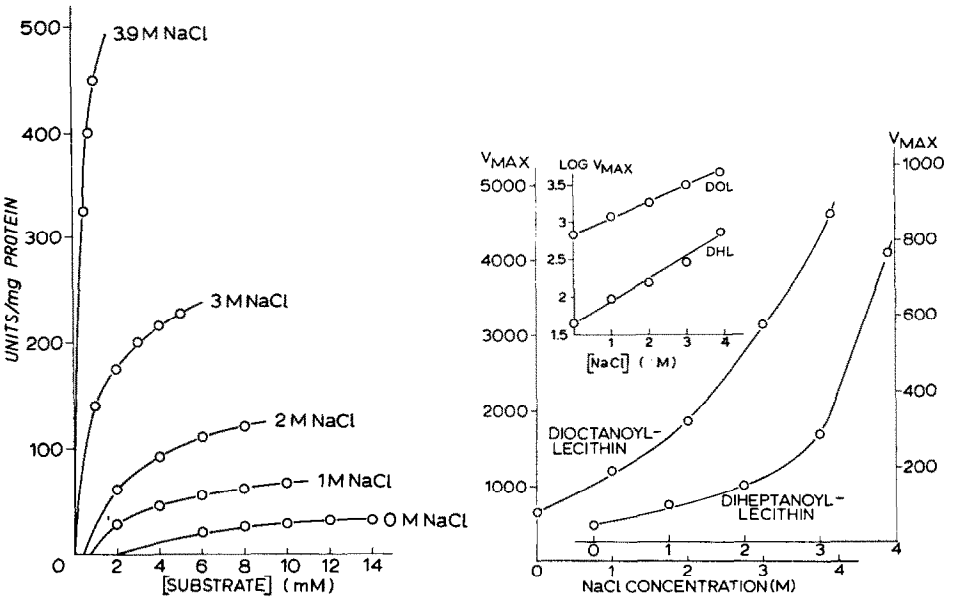


Fig. 9. Influence of varying NaCl concentrations on the hydrolysis of diheptanoyllecithin by phospholipase A. Assay conditions: 0.5 mM Tris, 1 mM $CaCl_2$, and varying substrate concentrations. All measurements were performed at pH 7.0. NaCl concentrations are indicated in the figure. Substrate concentrations are given in moles/l of lecithin (micelles *plus* monomers), which allows observation of the change in CMC with increasing salt concentration.

Fig. 10. Plot of v_{max} values *versus* NaCl concentration obtained from hydrolysis measurements of diheptanoyl- and dioctanoyllecithin by phospholipase A. Assay conditions as described in Fig. 9. v_{max} (at $[Ca^{2+}]$ = 1 mM) values were obtained from Lineweaver–Burk plots in which $[S]$ was corrected for the CMC. Abscissa at the left gives the scale for dioctanoyllecithin and abscissa at the right that for diheptanoyllecithin. Insert: log v_{max} values plotted *versus* NaCl concentration. Straight lines indicate an exponential increase of the v_{max} with increasing salt concentration. DHL = diheptanoyllecithin; DOL = dioctanoyllecithin.

Burk plots of the curves from Fig. 9. A plot of these v_{max} values as a function of the NaCl concentration is given in Fig. 10 for two substrates. For both compounds an exponential increase in activity was observed and no limit could be reached. Compare the insert in Fig. 10 where a linear relationship exists between log v_{max} and the NaCl concentration. One may wonder whether this salt effect is primarily caused by alteration in enzyme conformation and activity or, which is more plausible, by changes in the lipid–water interfaces. Kinetic measurements of phospholipase A on monolayers of dioctanoyllecithin at a fixed low pressure showed no such increase in activity whether NaCl (2 M) was present or not. Since high salt concentration does not influence the lecithin monolayer, these results seem to indicate that the high ionic strength does not strongly modify the enzyme structure or properties.

Finally Table II shows the salt dependence on the kinetic parameters K_s and $K_{Ca^{2+}}$. Small differences in enzyme conformation caused by dehydration might be responsible for the slightly higher affinity between enzyme and substrate (K_s) at increasing salt concentrations. Taking into account the fact that generally purely ionic interactions will decrease in solutions of increasing ionic strength (*cf.* the raise of $K_{Ca^{2+}}$ with increasing NaCl concentrations), the small shift in K_s values might indicate

TABLE II

INFLUENCE OF INCREASING NaCl CONCENTRATIONS ON KINETIC PARAMETERS OF PHOSPHOLIPASE A WITH A SHORT-CHAIN LECITHIN AS SUBSTRATE

Assays were performed at pH 7.0 (0.5 mM Tris). Values for v_{max} (μmoles/mg per min) and K_s (mM) were obtained from Lineweaver–Burk plots with diheptanoyllecithin as substrate at varying $CaCl_2$ concentrations. K_s is expressed in substrate concentration present in the form of micelles. $K_{Ca^{2+}}$ values were obtained from double reciprocal plots of activity *versus* Ca^{2+} concentration at 5 different substrate concentrations. At every NaCl concentration a common intersection on the abscissa was obtained.

NaCl Concn. (M)	v_{max} *(at* $[Ca^{2+}] = \infty$*)*	K_s *(mM)*	$K_{Ca^{2+}}$ *(mM)*
0	53.5	4.6	0.2
1	322	4.4	2
2	1600	2.3	10
3	4300	1.0	10

a considerable contribution of the Van der Waals interactions forces to the binding energy between enzyme and substrate*. It is difficult to provide an explanation for the 80 fold increase in v_{max} ($[Ca^{2+}]$ and $[S]$ = infinite) in the range from 0 to 3 M NaCl. These high salt concentrations will undoubtedly change the parameters of both the lipid–water interface and the solvent. For instance, the water structure in and surrounding the micellar surface will be altered, the charge-charge interactions in the interface due to the polar head groups will diminish, and the dielectric constant of the solvent will be considerably lowered. More physicochemical studies on the salt-induced changes of such interfaces have to be done before a correlation with enzyme activity can be made.

On the other hand, from a practical point of view, the salt induced increase in activity might be useful to extend our knowledge about other lipolytic enzymes. Not only the pancreatic phospholipase A becomes extremely active at high ionic strength, but also, for instance, the action of pancreatic lipase on lecithins is greatly enhanced. This enzyme which specifically cleaves fatty acid ester bonds in the 1-position of phospholipids in a non-stereospecific way has been used for preparative purposes to obtain 2-acyllysolecithins[40]. However, its action on phospholipids without the addition of salt is slow, and the prolonged incubation conditions needed for that reason may cause acyl migration. In the presence of high salt concentrations, lecithins are degraded very effectively by lipase and the enzymatic hydrolysis can be followed by pH-stat techniques.

CONCLUSIONS

Although pancreatic phospholipase A is able to hydrolyze slowly substrate molecules in monomeric dispersions, the enzyme attacks substrate molecules which are organized in certain lipid–water interfaces at a much higher rate. Experimental difficulties encountered in the kinetic measurements in the monomer region prevent us so far from explaining the differences in terms of binding (K_s) or catalysis (v_{max}). Slightly different substrates, such as a homologous series of lecithins, produce different lipid–water interfaces (size of the micelle, area per molecule in the interface, *etc.*).

* It should be remarked once more, however, that comparison of K_s values obtained with different interfaces is not justified.

Therefore the affinity constants between enzyme and even very similar substrates cannot be compared with each other. The observed large differences in the v_{max} values with such substrates cannot be explained by differences in chemical reactivity. One of the interfacial parameters which might govern the maximal rate of hydrolysis is the area per molecule, or charge density in the lipid–water interface.

The enzyme absolutely requires Ca^{2+} for activity and this generally found characteristic property has been explained in terms of a specific bridge of the metal between the enzyme and an anionic site of the substrate[41]. Although such a bridge mechanism cannot be precluded for more acidic phospholipids, the kinetic results obtained with lecithins under the conditions used do not support this function of the metal and rather point to a mutually independent binding of metal and substrate to the enzyme. In this respect it should be remarked that direct binding experiments failed to demonstrate any affinity of Ca^{2+} for short-chain lecithins, which is in agreement with the results of ROJAS AND TOBIAS[42] and of HAUSER AND DAWSON[43].

The fundamental role of certain interfacial parameters in the highly effective hydrolysis of lipids by lipolytic enzymes is still far from being understood. It is hoped that a simultaneous investigation of the enzyme kinetics by "bulk" methods and monolayer techniques will aid in a better understanding of these lipoprotein interactions.

ADDENDUM

Approximated calculations of the area/molecule in micelles of short-chain lecithins

Using the spherical micelle model (Harkin's micelle) and the micellar molecular weight as determined by light scattering, the surface area occupied by each lecithin molecule can be calculated as follows: Light-scattering data indicated that micelles of dihexanoyllecithin at 0.1 M NaCl at 25° consist of 25 monomers. With the volumes of the polar headgroup (336 Å^3), the CH_3-group (54 Å^3) and the CH_2-group (27 Å^3) as determined by GULIK-KRZYWICKI *et al.*[44], the total volume of the spherical micelle, consisting of 25 monomers (16500 Å^3) enables us to calculate the radius of the micelle to be 15.18 Å. From the total surface of the micelle ($4\pi r^2 = 3140$ Å^2) and the number of monomers (25) per micelle, the area occupied by one dihexanoyllecithin molecule is found to be 125 Å^2. A similar calculation for diheptanoyllecithin (about 45 monomers per micelle) gives an area per molecule of 100 Å^2.

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