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A Kinetic Study of the Phospholipase A_2 (*Crotalus adamanteus*) Catalyzed Hydrolysis of 1,2-Dibutyryl-*sn*-glycero-3-phosphorylcholine†

Michael A. Wells

ABSTRACT: A detailed kinetic analysis of the *Crotalus adamanteus* phospholipase A_2 catalyzed hydrolysis of dibutyryllecithin has been carried out. The concentration of dibutyryllecithin chosen insured that the substrate was in the monomeric state. Initial velocity patterns as a function of Ca^{2+} and dibutyryllecithin concentration, as well as inhibition studies with Ba^{2+}, are consistent with an ordered addition of reactants to the enzyme. Ca^{2+} adds first and the K_{iCa} is 4×10^{-5} M. Dibutyryllecithin adds second with a $K_{DBL} = 0.032$ M. Product inhibition studies and dead-end inhibition studies with butryramide are consistent with an ordered release of products. The fatty acid is released first from the enzyme and the lysolecithin is released second. The K_i for lysobutyryllecithin is 0.075 M and for butyramide it is 0.2 M. Evidence is presented that butyric acid inhibits as butyrate at pH 8.0 and must be considered as a dead-end inhibitor. The pH–activity profile of the enzyme shows a maximum at pH 8.0–8.5. K_i for Ca^{2+} is independent of pH in the range of 7.0–9.0. V_m/K_{DBL} is nearly independent of pH in the same range. V_m is, however, pH dependent and evidence is presented that a group with a pK_{app} near 7.6 in the enzyme–substrate complex is involved in catalysis.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolysis of the fatty acid esterified to the number two position of 1,2-diacyl *sn*-phosphoglycerides, and has been used for several years to determine the positional distribution of fatty acids in phospholipids. Several reports have appeared in which various aspects of the enzymatic reaction have been investigated.

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Van Deenen and de Haas (1963), reported the substrate specificity of the *Crotalus adamanteus* enzyme using whole venom and substrates as aqueous buffered emulsions. Although no kinetic data were reported they were able to establish the following points. (1) The enzyme is stereospecific and hydrolyzes only the fatty acid from 1,2-diacyl *sn*-glycerophospholipids. (2) β-Lecithins were hydrolyzed in a stereospecific manner, also, with only one isomer being attached. (3) Glycol analogs were also hydrolyzed. (4) They concluded that the minimum structural requirements for an active substrate were

Although the use of emulsified substrates can give qualitative information, obtaining quantitative kinetic data is complicated by several factors as pointed out by Roholt and Schlamowitz (1961). The aggregate nature of the substrate introduces uncertainties concerning the actual concentration of the substrate, and if more than one aggregate form are present (Tinker and Pinteric, 1971), which may have different affinities for the enzyme, further complications are introduced. Dawson (1963a) has shown that the activity of emulsified substrates is a critical function of the state of aggregation, and studies on mixed acid lecithins containing one short- and one long-chain fatty acid (Bird *et al.*, 1965; Attwood *et al.*, 1965) also indicate that the physical nature of the substrate profoundly influences its activity. Thus, kinetic experiments will depend on the nature of the substrate and the manner in which it is emulsified.

Further complications can arise due to the influence of products on the aggregate nature of the substrate. Dawson (1963b) has demonstrated that the accumulation of fatty acid in the substrate aggregate can inhibit the enzyme, but whether this is due to specific product inhibition and/or to an increased negative charge on the surface of the aggregate is unknown (Roholt and Schlamowitz, 1961). Thus, kinetic experiments on product inhibition are complicated by unknown influences on the aggregate nature of the substrate.

In order to overcome these difficulties Roholt and Schlamowitz (1961) studied the hydrolysis of dihexanoyl lecithin.[1] They showed that hydrolysis could occur when the substrate was in the monomeric form (2–5 mM). They presented evidence for the existence of an enzyme–Ca^{2+} intermediate, but could not demonstrate inhibition by hexanoic acid. They found that longer chain lysolecithins stimulated the reaction, although they did not use the lysolecithin which was a product of the reaction. Very recently de Haas *et al.* (1971) have reported extensive studies on the hydrolysis of short-chain lecithins in micellar solution using the pancreatic phospholipase A_2. A more complete analysis of this important paper will be deferred to the Discussion section of this paper.

Wells (1971a), using the highly purified enzymes from *Crotalus adamanteus* venom, has presented evidence that the hydrolysis proceeds by *O*-acyl cleavage, but was unable to obtain any evidence for the existence of an acyl-enzyme intermediate. In order to further characterize the reaction it was clear that a detailed kinetic study was necessary. The limitations of using aggregate substrates, pointed out above, dictated the use of monomeric substrates as first proposed by Roholt and Schlamowitz (1961). However, in order to increase the concentration at which the substrate exists as monomer, a shorter chain lecithin, dibutyryllecithin, was used. This paper presents the results of this study.

Materials and Methods

Materials

The α and β forms of phospholipase A_2 were isolated from *Crotalus adamanteus* venom as described by Wells and Hanahan (1969). Identical results were obtained with both forms of the enzyme and no distinction will be made as to which results were obtained with which form of the enzyme. Stock solutions were prepared in distilled water and thoroughly dialyzed against distilled water and then against highly purified water (see below). The pH of the stock solution was adjusted to about 8.0 with NaOH and the protein concentration determined by measuring the absorbance at 280 nm.

1,2-Dibutyryl-*sn*-glycero-3-phosphorylcholine was prepared as described previously (Wells, 1971b). The purified product analyzed for 7.55% phosphorus (theoretical % P = 7.50) and had $[\alpha]_{546}^{22} = +13.0°$ (*c* 1.8, chloroform–methanol, 1:1, v/v) or $+5.1°$ (*c* 4.1, H_2O). *sn*-Glycero-3-phosphorylcholine was prepared from egg-yolk lecithins by the method of Brockerhoff and Yurkowski (1965). The cadmium chloride complex was converted to the free diester by treatment with a mixed-bed resin of Bio-Rex 70 and AG3-X4 (Bio-Rad Laboratories, Richmond, Calif.). The purified product had $[\alpha]_{546}^{22} = -3.0°$ (*c* 2.5, H_2O).

1-Butyryl-*sn*-glycero-3-phosphorylcholine was prepared in the following manner. 1-Butyryl-2-palmitoyl-*sn*-glycero-3-phosphorylcholine was prepared by the method of Bird *et al.* (1965) for the preparation of the 1-butyryl-2-oleoyl compound. The final product was purified on a column of silicic acid (silicAR CC-4 Mallinckrodt Chemical Works, St. Louis, Mo.) as previously described (Saunders and Wells, 1969). The purified material analyzed for 5.42% phosphorus (theoretical % P = 5.40), and had $[\alpha]_{546}^{22} = +9.2°$ (*c* 6.25, chloroform–methanol, 1:1, v/v). This product was reacted with purified phospholipase A_2 in diethyl ether (Wells and Hanahan, 1969), using 1.5 mmoles of the phospholipid and 100 μg of enzyme in a volume of 25 ml. After standing overnight the solvent was removed *in vacuo*, and the residue, dissolved in 5 ml of chloroform, was applied to a 50-g column of silicic acid (SilicAR CC-7, Mallinckrodt Chemical Works). The column was eluted in succession with chloroform (1 l.), chloroform–methanol (1:1, v/v, 1 l.), and methanol–water (9:1, v/v, 4 l.). The last solvent was collected in fractions and the fractions containing the product (as determined by phosphorus analysis) were combined and dried *in vacuo*. The slightly yellow material was dissolved in methanol (25 ml) and treated with 0.5 g of Darco G-60 charcoal. After removal of the charcoal the now colorless material amounted to 1.07 mmoles (71% yield) and analyzed for 9.05% phosphorus (theoretical % P = 8.97%), and had $[\alpha]_{546}^{22} = -0.8°$ (*c* 3.5, H_2O).

1-Butyryl-*sn*-glycero-3-phosphorylcholine was also prepared enzymatically in the following manner. Dibutyryllecithin (500 μmoles) was reacted with 100 μg of enzyme in 5 ml of 10^{-3} M $CaCl_2$. The pH of the solution was maintained

[1] According to the tentative rules for lipid nomenclature (IUPAC) these compounds should be named 1,2-diacyl-*sn*-glycero-3-phosphorylcholines. For simplicity they will be abbreviated dibutyryllecithin, etc., 1-acyl-*sn*-glycero-3-phosphorylcholines will be called lysolecithins.

at 7.0 by the addition of NaOH. After 3 hr, approximately 60% of the theoretical amount of base had been consumed. The reaction mixture was lyophilized and the products were purified on silicic acid as described above. Two phosphorus-containing peaks were isolated from the column. The first which amounted to 225 μmoles had $[\alpha]_{546}^{22} = +5.0°$ (H_2O) and was unreacted starting material. The second which amounted to 250 μmoles had $[\alpha]_{546}^{22} = -0.3°$ (H_2O) and was the desired product. The recovery of phosphorus from the column was 95%.

Water was purified by redistilling distilled water in a two-stage quartz still. Buryramide (Eastman Kodak Co., Rochester, N. Y.) was recrystallized twice from methanol–ether and had a melting point of 115–116° uncor. Butyric acid (Eastman Kodak Co.) was redistilled before use. All other chemicals were reagent grade and used without further purification. All the phosphorylcholine-containing compounds were thoroughly deionized by passing through a mixed-bed resin of Bio-Rex 70 and AG3-X4. Optical activity was measured in an OLD 4 (Carl Zeiss, Inc., New York).

Methods

Enzyme Assays. Hydrolysis of substrate was measured using a Radiometer (Copenhagen) TTA31 microtitration assembly. The glass buret unit supplied by the company was replaced with a 50-μl syringe (Hamilton Co., Inc., Whittier, Calif.), which was connected by 0.025-in. i.d. Teflon tubing to the reaction vessel. Full displacement of the syringe drive motor corresponded to the addition of approximately 20 μl of titrant. The reaction was carried out in a total volume of 1.0 ml at 37° in an atmosphere of N_2. The pH was maintained by the addition of 0.025 M NaOH. After all reactants except the enzyme were added to the vessel, a baseline was recorded for 5 min. The reaction was initiated by the addition of enzyme, generally 10 μg in 50 μl, and the rate was followed for 5 min. The amount of base added during the reaction was less than 10 μl so that dilution was negligible. The amount of substrate consumed was between 0.5 and 1.0% during the incubation. The reaction was linear for at least 30 min and up to at least 5% hydrolysis, so that under all conditions reported only initial velocities were observed. Baseline corrections were negligible except at the highest pH's studied. Duplicate assays were reproducible within 5–10%. The reaction mixture consisted of substrate, $CaCl_2$, enzyme, and other additions when appropriate. Under no circumstances were any buffering agents added. Butyric acid was added as its sodium salt. Unless otherwise stated, all data were obtained at pH 8.0.

For certain purposes a spectrophotometric assay was used. In this assay a pH indicator was used to buffer the reaction at pH 8.0 and the rate of production of the acid form of the indicator was used to measure the rate of hydrolysis of the substrate. The sample cuvet contained 1.0 ml of a solution which was 50 mM substrate, 50 μM $CaCl_2$, and 0.5 mM bromothymol blue. This solution was adjusted to pH 8.0 with NaOH. The blank cuvet contained the same amount of substrate and indicator, but 50 μM $BaCl_2$ was substituted for the $CaCl_2$ in order to inhibit the enzymatic reaction. This solution was also adjusted to pH 8.0. The absorbance at 475 nm, which is the absorption minimum of the basic form of the indicator, and is near the absorption maximum of the acid form of the indicator (440 nm) was measured on the 0.1-absorbance slide-wire of a Cary Model 15 spectrophotometer. After balancing the two solutions to read 0.00 absorbance, 10 μl of enzyme was placed in the reference cuvet, and the reference chamber was closed. With the sample cuvet in place in the sample chamber, 10 μl of enzyme solution were added and rapidly mixed, the chamber closed, and the recorder started. The chart speed was 1 division/5 sec. A change in absorbance of 0.01/5 sec corresponds to a rate of 0.05 μequiv of fatty acid released per min. The reaction rate was linear for a change of absorbance from 0.00 to 0.1 or for about 2 min. No significant reaction was observed in the absence of added enzyme. Once the reaction had been started, further additions could be made by stopping the recorder, opening the sample chamber, adding the desired component, mixing, closing the chamber, and restarting the recorder. These additions could be accomplished in about 5 sec.

The reaction products were analyzed on thin layers of cellulose, Polygram Cel-300 (Brinkman Instruments, Inc., Westbury, N. Y.), using the solvent system isopropyl alcohol–concentrated NH_4OH–H_2O (7:1:2, v/v). The phosphorus-containing compounds were detected by the spray of Hanes and Isherwood (1949). R_F values were as follows: dibutyryllecithin, 0.75; lysobutyryllecithin, 0.60 and glycerophosphorylcholine, 0.50.

Calcium ion activity was measured using a calcium specific electrode (Orion Research, Inc., Cambridge, Mass.) and a pH meter 4 (Radiometer, Copenhagen). Substrate molecular weights were determined using a Hewlett-Packard Model 302B vapor pressure osmometer and mannitol as a standard. Kinetic data were analyzed by the double-reciprocal plotting method of Lineweaver and Burk (1939). Kinetic equations were derived by the method of King and Altman (1956) as described for bireactant mechanisms by Cleland (1963a–c). Appropriate equations will be found in the appendix to this paper.

Results

General Properties of the Reaction System. It must be emphasized at the outset that the results reported here were obtained only after painstaking attention to the purification of all materials. For example, the distilled water available in this laboratory was found to contain variable amounts of volatile amines which complicated studies using Ca^{2+}. Reproducible results were possible only when quartz-distilled water was used. The high polarity of the dibutyryl- and lysobutyryllecithins requires the use of methanol or methanol–water mixtures during silicic acid chromatography, and it was found that these polar solvents elute inorganic materials from the silicic acid which were strongly inhibitory. These inhibitors could be removed by passing the dibutyryl- or lysobutyryllecithins through a mixed-bed ion-exchange resin, however, not all ion-exchange resins were satisfactory. In particular sulfonic acid based resins were found to release material which inhibited the reaction. These examples will suffice to point out the types of problems which were encountered.

The rate was linear with respect to enzyme concentration in the range of 2.5–50 μg of enzyme using a concentration of dibutyryllecithin which varied from 10 to 50 mM. Under the conditions most often used, 10 μg of enzyme, the rate was linear for more than 30 min. Conditions were chosen such that less than 1% of the substrate was consumed during the assay. That the enzyme was indeed acting as a phospholipase A_2 under these conditions was established in the following manner. The only phosphorus containing compounds detected in the reaction mixture were unreacted dibutyryllecithin and lysobutyryllecithin, whereas glycerophosphoryl-

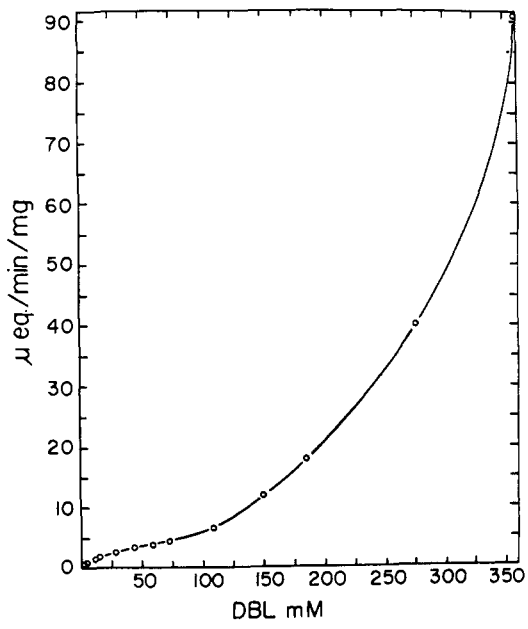


FIGURE 1: Initial velocity as a function of dibutyryllecithin concentration. Reaction carried out at pH 8.0, and 37° in the presence of 10^{-3} M $CaCl_2$.

choline was never detected. In one large-scale experiment the lysobutyryllecithin was isolated and shown to have the same optical activity as synthetic material (see Materials section), thus establishing that the enzyme proceeds in the expected stereospecific manner.

Monomeric Nature of Dibutyryllecithin. It was necessary to determine the concentration range in which this substrate could be used without exceeding the critical micelle concentration. Figure 1 is a typical velocity *vs.* substrate plot at one concentration of Ca^{2+}. It can be seen that saturation is approached between 75 and 100 mM and then there is an exponential rise in activity above 100 mM. Figure 2 is a double-reciprocal plot of the data in Figure 1 and shows a linear portion below 75 mM and strong deviation from linearity above 100 mM. Molecular weight determinations by vapor pressure osmometery in the concentration range of 10–50 mM gave a value of 410 (±50) which compares favorably to the theoretical value of 414. On the basis of these data it was concluded that at concentrations up to 50 mM dibutyryllecithin is present in solution as monomers, and all experiments were conducted in this concentration range. These data should not be interpreted as constituting a determination of the critical micelle concentration, but only to show that the substrate is monomeric in the concentration range used for these studies.

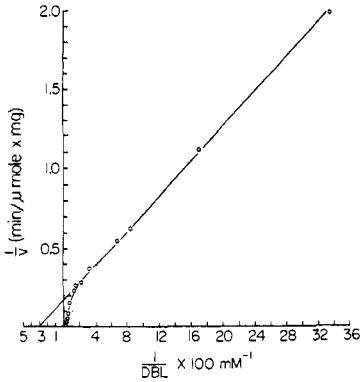


FIGURE 2: Double-reciprocal plot of initial velocity as a function of dibutyryllecithin concentration. The data from Figure 1 have been replotted in double-reciprocal form.

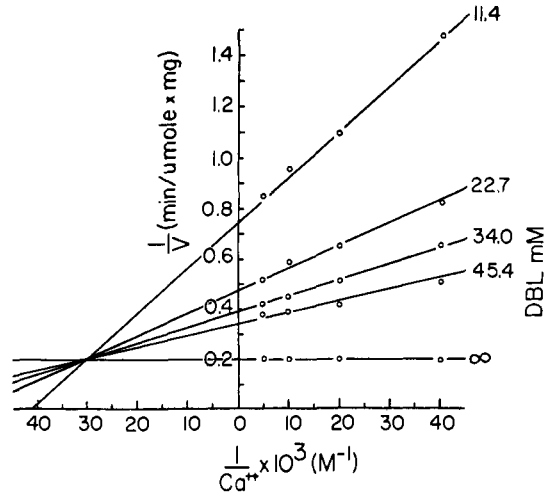


FIGURE 3: Double-reciprocal plot of initial velocity as a function of Ca^{2+} concentration at various fixed levels of dibutyryllecithin. The numbers to the right of the lines indicate the fixed level of dibutyryllecithin. The data for infinite dibutyryllecithin is taken from Figure 4.

Initial Velocity Studies. Figure 3 shows double-reciprocal plots of velocities as a function of Ca^{2+} concentration at various fixed levels of dibutyryllecithin. Figure 4 shows double-reciprocal plots of velocities as a function of dibutyryllecithin concentration at various fixed levels of Ca^{2+}. Figure 5 is a replot of slopes and intercepts from Figure 3 as a function of dibutyryllecithin concentration, and Figure 6 is a replot of slopes and intercepts from Figure 4 as a function of a Ca^{2+} concentration.

These data are consistent with a mechanism which in-

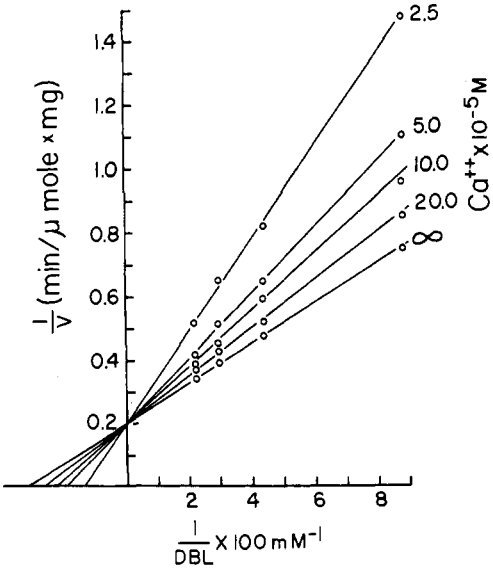


FIGURE 4: Double-reciprocal plot of initial velocity as a function of dibutryryllectithin concentration at various fixed levels of Ca^{2+}. The numbers to the right of the lines indicate the fixed level of Ca^{2+}. The data for infinite Ca^{2+} is taken from Figure 3.

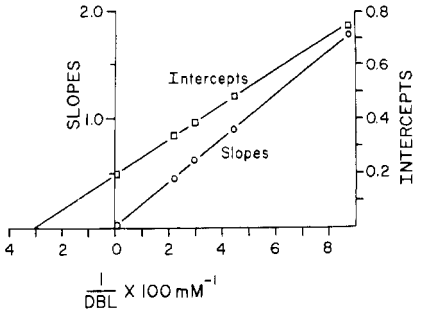


FIGURE 5: Replot of slopes and intercepts from Figure 3 as a function of the fixed level of dibutyryllecithin.

TABLE I: Kinetic Constants[a] for Phospholipase A_2 Catalyzed Hydrolysis of Dibutyryllecithin at pH 8.0 and 37°.

$K_{iCa} = 4 \times 10^{-5}$ M	$K_{DBL} = 0.032$ M	$K_{iLBL} = 0.075$ M
$K_{iBA} = 0.05$ M	$K_{iBN} = 0.20$ M	$K_{iBN} = 0.13$ M
$K_{iBa} = 3 \times 10^{-5}$ M	$(k_5 + k_7)/k_5 = 1.5$	$k_2 \geq 1$ sec^{-1}
$V_m = 5$ μequiv/min per mg		Turnover number = 2.5 moles/sec
$V_m/K_{DBL} = 78$ sec^{-1} mole^{-1}		

[a] See Appendix for definition of constants. Abbreviations: BA = butyric acid, BN = butyramide, DBL = dibutyryllecithin, LBL = lysobutyryllecithin.

volves ordered addition of reactants with Ca^{2+} adding first and dibutyryllecithin adding second (Cleland, 1970). In Figure 5 the intercept replot intersects the $1/v$ axis at $1/V_m$ and the 1/DBL axis at $-1/K_{DBL}$. The ratio of the slopes of the slope replot and intercept replot is $K_{iCa}{}^{2+}$. In Figure 6, the intercept plot also intersects the $1/v$ axis at $1/V_m$, while the slope replot intersects the $1/(Ca^{2+})$ axis at $-1/K_{iCa}{}^{2+}$. The values for these constants are presented in Table I along with values for the turnover number of the enzyme and V_m/K_{DBL}, which is the apparent first-order rate constant for the reaction of the enzyme with dibutyryllecithin at very low concentrations of dibutyryllecithin.

The possibility that a Ca^{2+}–dibutyryllecithin complex was the substrate for the reaction was eliminated in the following experiment. No significant difference was detected when the Ca^{2+} activities of solutions of $CaCl_2$, which varied in concentration from 5×10^{-5} to 1×10^{-3} M, were measured either in the presence of 25 mM dibutyryllecithin or NaCl of the same ionic strength. This would indicate that less than 0.1% of the dibutyryllecithin was present as the Ca^{2+} complex. This conclusion is also supported by studies on Ca^{2+} binding to monolayers of lecithin (Rojas and Tobias, 1965; Hauser and Dawson, 1967) and aqueous dispersions of lecithin (Barton, 1968).

Ray and Roscelli (1966) have shown that the apparent ordered addition of Mg^{2+} to phosphoglucomutase is a consequence of the slow rate of dissociation of the Mg^{2+} from the enzyme. Using their all-or-none assay approach an attempt was made to measure the rate of dissociation of Ca^{2+} from phospholipase A_2. The experiment was carried in two ways using the spectrophotometric assay. The first experiment consisted of initiating a reaction in the presence of 50 μM Ca^{2+}, and then after 15 sec interrupting the reaction, adding 10 μl of a saturated solution of $BaCl_2$, and measuring the decay of the enzymatic activity. The other approach involved preparing the substrate solution with 10 μl of a saturated solution of $BaCl_2$, then adding the enzyme (10 μl) which was prepared in the presence of 10^{-3} M $CaCl_2$, and measuring the decay of the enzyme activity. In both cases the time elapsed between the addition of $BaCl_2$ in expt 1 or the addition of enzyme in expt 2 was about 5 sec, yet no enzymatic activity was detected. This would indicate that Ca^{2+} is released from the enzyme with a rate constant greater than or equal to 1 sec^{-1}.

Product Inhibition Studies. Figure 7A shows double-reciprocal plots of velocities *vs.* Ca^{2+} concentration at one level of dibutyryllecithin and in the presence of lysobutyryllecithin or butyric acid. Figure 7B shows similar data as a function of dibutyryllecithin concentration at one level of Ca^{2+}. Both lysobutyryllecithin and butyric acid gave uncompetitive inhibition toward Ca^{2+} and competitive inhibition toward dibutyryllecithin.

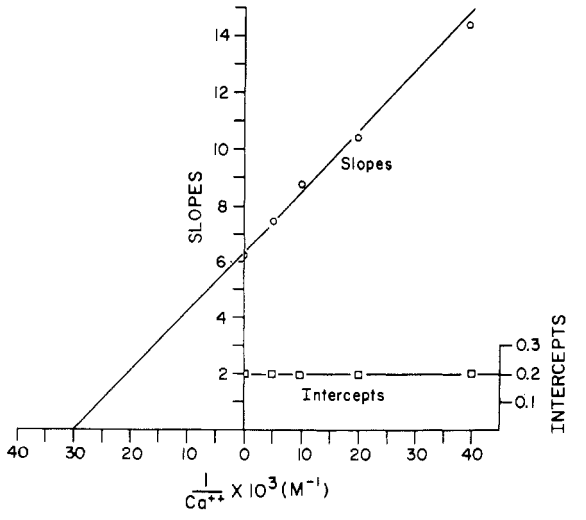


FIGURE 6: Replot of slopes and intercepts from Figure 4 as a function of the fixed level of Ca^{2+}.

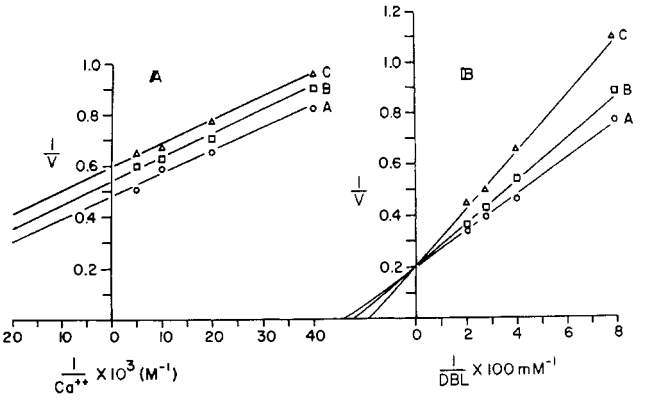


FIGURE 7: Inhibition by lysobutyryllecithin and butyric acid. Panel A: double-reciprocal plots of initial velocity as a function of Ca^{2+} concentration in the presence of 25 mM dibutyryllecithin. Curve A was obtained with no further additions, curve B with the addition of 25 mM lysobutyryllecithin and curve C with the addition of 25 mM butyric acid. Panel B: double-reciprocal plots of intial velocity as a function of dibutyryllecithin concentration in the presence of 10^{-3} M Ca^{2+}. Curve A was obtained with no further additions, curve B with the addition of 25 mM lysobutyryllecithin, and curve C with the addition of 25 mM butyric acid.

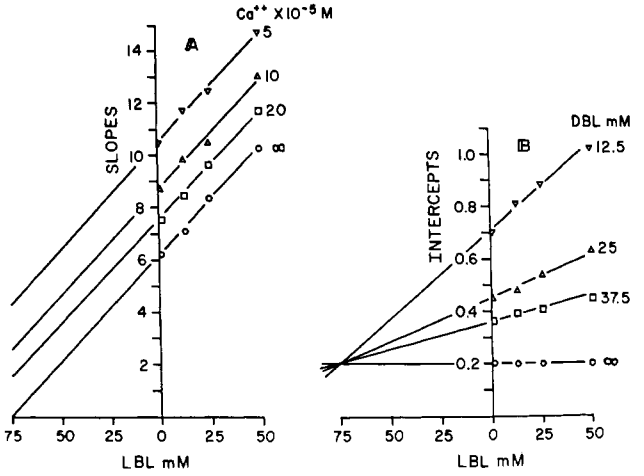


FIGURE 8: Characterization of inhibition by lysobutryllecithin. Panel A: inhibition *vs.* Ca^{2+}. These data were obtained by carrying out initial velocity experiments as a function of dibutyryllecithin concentration at various fixed levels of Ca^{2+} and lysobutyryllecithin. Data such as that shown in Figure 3 were obtained. The slopes of the lines at various fixed levels of Ca^{2+} are replotted in this figure as a function of the lysobutyryllecithin concentration. The numbers to the right of the lines indicate the level of Ca^{2+}. Panel B: inhibition *vs.* dibutyryllecithin. These data were obtained by carrying out initial velocity experiments as a function of Ca^{2+} concentration at various fixed levels of dibutyryllecithin and lysobutyryllecithin. Data such as shown in Figure 4 were obtained. The intercepts of the lines at various fixed levels of dibutyryllecithin are replotted in this figure as a function of the lysobutyryllecithin concentration. The numbers to the right of the lines indicate the dibutyryllecithin concentration.

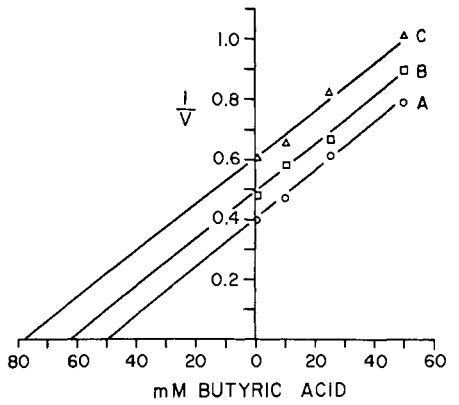


FIGURE 9: Characterization of the inhibition by butyric acid. Reciprocal velocity *vs.* butyric acid concentration. Experiments were carried out in the presence of 10^{-3} M Ca^{2+} and 25 mM dibutyryllecithin and the indicated amount of butyric acid. Curve A, no further additions; curve B with 25 mM lysobutyryllecithin added; and curve C with 50 mM lysobutyryllecithin added.

Further characterization of the inhibition by lysobutyryllecithin is found in Figure 8. Figure 8A demonstrates that the inhibition *vs.* Ca^{2+} is linear and that the extent of inhibition depends on the concentration of Ca^{2+}, but that ∞ Ca^{2+} cannot overcome the inhibition. The intersection of the ∞ Ca^{2+} line with the lysobutyryllecithin axis is $-K_i$ for lysobutyryllecithin. Figure 8B demonstrates that inhibition *vs.* dibutyryllecithin is also linear and that the inhibition is overcome at ∞ dibutyryllecithin. The inhibition curves obtained at various levels of dibutyryllecithin intersect at a common point equal to $-K_i$ for lysobutyryllecithin. The value for this constant is found in Table I.

There is no doubt that lysobutyryllecithin is acting as a product inhibitor. However, a serious question can be raised as to the interpretation of the inhibition caused by butyric acid. The simplest interpretation of the stoichiometry of the reaction would predict that the acyl group would be released as butyric acid, RCOOH, but at pH 8.0 this would be instantaneously converted to butyrate, $RCOO^-$. On the other hand, possible mechanisms could be written in which the butyrate and the proton would be released independently from the enzyme. If the released acyl group is butyric acid then inhibition studies carried out with butyrate are open to ambiguity since there is no assurance that these two species would behave in the same manner. The enzyme is not active at pH's low enough to allow appreciable concentrations of butyric acid to be present, and so more indirect methods must be used to resolve this question.

There are three ways in which butyrate could cause inhibition. It could be acting as a product inhibitor, a dead-end inhibitor, or by complexing Ca^{2+} and lowering the effective concentration of Ca^{2+} in the reaction mixture. If butyrate were indeed acting as a product inhibitor, then inhibition studies carried out in the presence of both butyrate and lysobutyryllecithin should demonstrate synergistic inhibition, since there should be a binding site for each product. It may be possible that the synergistic inhibition would not be demonstrable, but at least one should be able to demonstrate independent binding sites for the two products. Figure 9 shows the results of such an experiment. The inhibition curves are parallel which suggests that butyrate and lysobutyryllecithin are mutually exclusive inhibitors and compete for the same site on the enzyme (Cleland, 1970). This result would not be consistent with butyrate acting as a product inhibitor. The K_i for butyrate can be determined from the inhibition curve obtained in the absence of lysobutyryllecithin and its value is presented in Table I.

In order to test the possibility that butyrate inhibition was due to complex formation with Ca^{2+}, Ca^{2+} activities were measured in the presence of 0.1 M sodium butyrate, 0.1 M sodium chloride, and with no additions. The Ca^{2+} concentration varied from 10^{-5} to 10^{-3} M. The Ca^{2+} activity was lowered by the presence of sodium butyrate, but within experimental error this was an ionic strength effect, since identical activities were obtained in the presence of sodium chloride, which is not inhibitory. No more than 10% of the Ca^{2+} could be complexed by butyrate on the basis of these experiments. The log of the stability constant of calcium butyrate is 1.05 (Phillips and Williams, 1966), which would also suggest that the inhibition by butyrate is not due to complexing of Ca^{2+}.

Inhibition by Butyramide. It was considered possible that butyramide, $RCONH_2$, might act as an analog of butyric acid, RCOOH, in that it would not be ionized. Inhibition studies carried out in the presence of butyramide are presented in Figure 10. Butyramide is an uncompetitive inhibitor with respect to Ca^{2+} in the presence or absence of lysobutyryllecithin (Figure 10A). Butyramide is an uncompetitive inhibitor with respect to dibutyryllecithin, but is a noncompetitive inhibitor when lysobutyryllecithin is present (Figure 10B). When both butyramide and lysobutyryllecithin are present, ∞ dibutyryllecithin can overcome the inhibition due to the lysobutyryllecithin, but not that due to the butyramide.

Figure 11A shows that butyramide is a linear inhibitor with respect to dibutyryllecithin. The K_i calculated for butyramide is presented in Table I. Figure 11B contains a more detailed analysis of the inhibition by butyramide in the presence of

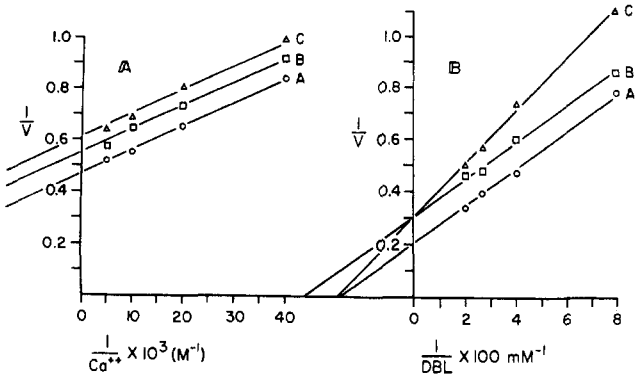


FIGURE 10: Inhibition by butyramide. Panel A: double-reciprocal plots of initial velocity as a function of Ca^{2+} concentration in the presence of 25 mM dibutyryllecithin. Curve A, no further additions; curve B, with 100 mM butyramide added; and curve C, with 100 mM butyramide and 25 mM lysobutyryllecithin added. Panel B: double-reciprocal plots of initial velocity as a function of dibutyryllecithin concentration in the presence of 10^{-3} M Ca^{2+}. Curve A, no further additions; curve B, with 100 mM butyramide added; and curve C with 100 mM butyramide and 25 mM lysobutyryllecithin added.

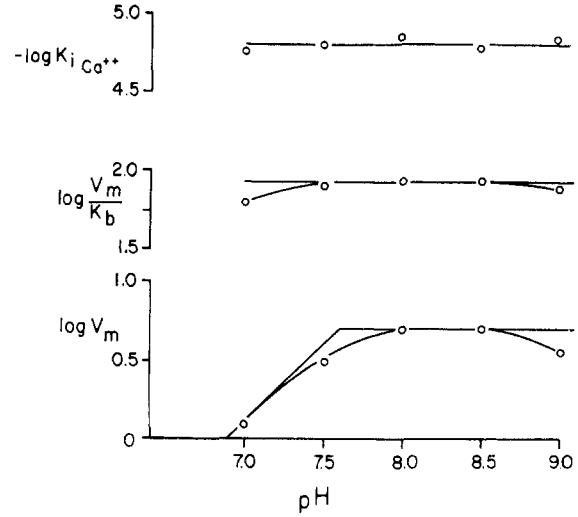


FIGURE 12: Kinetic constants as a function of pH. The kinetic constants were determined from experiments such as those described in Figures 3 and 4 carried out at the indicated pH's.

lysobutyryllecithin. The intersecting pattern is consistent with a synergistic inhibition of butyramide and lysobutyryllecithin. The interaction coefficient (Cleland, 1970) is +0.5.

Inhibition by Ba^{2+} and Other Cations. Ba^{2+} was shown to be a linear competitive inhibitor with respect to both Ca^{2+} and dibutyryllecithin. The K_i for Ba^{2+} is presented in Table I. Several other cations were found to be inhibitory and include Zn^{2+}, Cu^{2+}, Cd^{2+}, Fe^{3+}, Al^{3+}. Others such as Sr^{2+}, Mg^{2+}, and Mn^{2+} had little effect. Under no circumstances was it possible to find any cation which would replace Ca^{2+} in the enzymatic reaction. The inhibitory action of Cd^{2+} places additional emphasis on the need for extreme caution in the purification of substrates, since many synthetic reactions for making lecithins start with the $CdCl_2$ complex of glycerophosphorylcholine.

Inhibition by Glycerophosphorylcholine. Highly purified glycerophosphorylcholine prepared from the $CdCl_2$ complex by repeated treatment with mixed bed ion exchange resins was not inhibitory. Even when 0.5 M glycerophosphorylcholine was added to a reaction mixture which contained 0.01 M dibutyryllecithin, no inhibition was observed.

pH Dependence of the Reaction. Figure 12 shows the pH dependence of V_m, V_m/K_{DBL}, and K_{iCa}. The nonenzymatic rate of hydrolysis is too great and the enzymatic rate too low at pH 9.5 to obtain meaningful data. At pH 6.5 the rate is too low to obtain useful data, although at high substrate concentration it was possible to measure enzymatic activity. At pH 6.0 no enzymatic activity could be detected even at high substrate concentrations. The data in Figure 12 show that K_{iCa} is independent of pH in the range studied. V_m/K_{DBL} is nearly independent of pH, although it may decrease at the pH extremes studied. V_m is clearly pH dependent and suggests that a group with a $pK_{app} = 7.6$ in the enzyme–substrate complex is involved in the reaction.

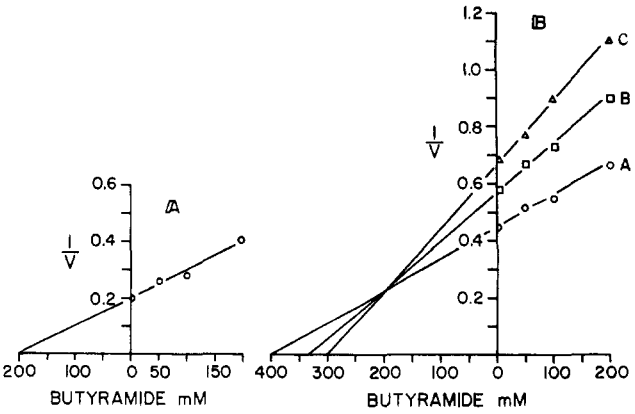


FIGURE 11: Characterization of inhibition by butyramide. Panel A: reciprocal of maximal velocities ($1/V$) obtained from double-reciprocal plots of velocities as a function of dibutyryllecithin at a fixed level of 10^{-3} M Ca^{2+} and the indicated amounts of butyramide. Panel B: reciprocal velocity as a function of butyramide concentration. All experiments were carried out in the presence of 25 mM dibutyryllecithin and 10^{-3} M Ca^{2+}. Curve A, no further additions; curve B, with 25 mM lysobutyryllecithin added; and curve C, with 50 mM lysobutyryllecithin added.

Discussion

The purpose of this work was to establish, as completely as possible within the limitations of the assay procedures available, the kinetic mechanism of phospholipase A_2 from *C. adamanteus* venom. The reasons for choosing a monomeric substrate are detailed in the introduction and need not be repeated here. In the following discussion reference will be made to kinetic equations derived for several possible mechanisms. The pertinent equations will be found in the appendix to the paper. Cleland (1970) has discussed some of the properties of equilibrium-ordered mechanisms, but the author has been unable to find full rate equations in the literature.

The data presented are consistent with a mechanism which involves ordered addition of Ca^{2+} and dibutyryllecithin. The addition of Ca^{2+} must precede the addition of dibutyryllecithin and is at thermodynamic equilibrium. Initial velocity patterns (Figures 3–6) are clearly consistent with this mechanism (Cleland, 1970). In addition, this mechanism predicts that a dead-end inhibitor which combines with E should exhibit competitive inhibition against both Ca^{2+} and dibutyryllecithin (eq A-10 and A-11 and Cleland, 1970). Ba^{2+} in fact shows such an inhibition pattern (Table II).

TABLE II: Inhibition Patterns for Some Bi-Ter Mechanisms.

Inhibitor	Variable Substrate: A, Unsaturated with B	A, Saturated with B	B, Unsaturated with A	B, Saturated with A
I. Ordered bi-ter (A-1)[a]				
P[b]	NC[d]	UC	NC	NC
Q[c]	UC	–	C	C
E + I	C	–	C	–
EA + I	UC	–	C	C
EAQ + I (Q = 0)	UC	UC	UC	UC
EAQ + I (+Q)	UC	UC	NC	NC
II. Rapid equilibrium ordered addition of reactants, rapid equilibrium random release of products (A-19).[a]				
P or Q	UC	–	C	C
E + I	C	–	C	–
EA + I	UC	–	C	C
EAQ + I (Q = 0)	–	–	–	–
EAQ + I (+Q)	UC	–	C	C
III. Ordered addition of reactants, random release of products (A-23).[a]				
P or Q	NC[e]	NC[e]	NC[e]	NC[e]
IV. Observed.				
Lysobutyryllecithin	UC	–	C	C
Butyric acid	UC	–	C	C
Butyramide (LBL = 0)	UC	UC	UC	UC
Butyramide (+LBL)	UC	UC	NC	NC
Ba^{2+}	C	–	C	–

[a] Refer to Appendix for illustration of mechanism. [b] P = first released product. [c] Q = second released product. [d] NC = noncompetitive, UC = uncompetitive, C = competitive, – = no inhibition. [e] Nonlinear inhibition.

The possibility must be considered that the addition of reactants appears to be ordered only because Ca^{2+} dissociates very slowly from the enzyme. Such a situation has been observed for phosphoglucomutase (Ray and Roscelli, 1966). In the case of phospholipase A_2 the Ca^{2+} is released with a rate constant which must be greater than 1 sec^{-1}, and so it has not yet been possible to directly confirm this possibility. In addition, kinetic studies at very low Ca^{2+} and high dibutyryllecithin concentrations do not provide any evidence that the addition of reactants is random. Therefore, under the conditions described in this study, the addition of reactants must proceed predominantly by an ordered mechanism.

The release of products could also be ordered (A-1), random (A-23), or equilibrium random (A-19). Considering for the moment only lysobutyryllecithin, the data in Figures 7 and 8, and Table II would be consistent with an ordered release of products with the lysobutyryllecithin being the second product release (A-8 and A-9, Table II) or a rapid equilibrium random release of products (Table II). The data are not consistent with a random release of products (A-23) since this mechanism predicts nonlinear noncompetitive product inhibition (Table II), nor with an ordered release of products if lysobutyryllecithin were the first product released (A-6 and A-7, Table II).

The data in Figure 7 for inhibition by butyrate would be consistent with a mechanism in which products are released in a rapid equilibrium random fashion (Table II). However, as pointed out above, the interpretation of the inhibition by butyrate is open to some question, since it is not entirely clear that this is representative of acyl group which is actually produced by the enzyme. In addition, the data presented in Figure 9 would suggest that butyrate and lysobutyryllecithin act as mutually exclusive inhibitors. This is presumptive evidence that butyrate is in fact not the acyl group which is released from the enzyme. However, it is possible that under the conditions used in the experiment the butyrate and lysobutyryllecithin are competing for some anionic site on the protein. The data in Figure 7 would also be consistent with butyrate acting as a dead-end inhibitor, which complexes with the enzyme–calcium intermediate, if the products are released in an ordered manner with lysobutyryllecithin being the second product released (A-12 and A-13).

In order to resolve this question inhibition studies were carried out with butyramide, which was considered an analog of butyric acid. The data in Figure 11b strongly suggest that butyramide complexes with enzyme–Ca^{2+}–lysobutyryllecithin intermediate. Furthermore, the inhibition pattern exhibited by butyramide (Figure 10A,B, Table II) is entirely consistent with an inhibitor which complexes with the enzyme–Ca^{2+} (second released product) intermediate in the ordered-product-release mechanism (A-14 and A-15). It should be noted that the rapid-equilibruim random product release mechanism predicts that an inhibitor which reacts with enzyme–Ca^{2+} (second released product) intermediate should not be inhibitory in the absence of added second product. The fact that butyramide does inhibit in the absence of added lysobutyryllecithin would tend to eliminate the rapid-equilibrium random mechanism. Table II contains the inhibition patterns predicted for the various mechanisms and the observed inhibition patterns. All the data are consistent with an ordered-product-release mechanism with butyric acid being the first released product and lysobutyryllecithin the second released product. The proposed mechanism for the overall reaction is ordered bi-ter (A-1), which can be represented as

$$\text{enzyme} + \text{Ca} \rightleftarrows \text{enzyme–Ca}$$

$$\text{enzyme} - \text{Ca} + \text{lecithin} \rightleftarrows \text{enzyme–Ca–lecithin}$$

$$\text{enzyme–Ca–lecithin} \rightleftarrows \text{enzyme–Ca–lysolecithin–fatty acid}$$

$$\text{enzyme–Ca–lysolecithin–fatty acid} \rightleftarrows \text{enzyme–Ca–lysolecithin} + \text{fatty acid}$$

$$\text{enzyme–Ca–lysolecithin} \rightleftarrows \text{enzyme–Ca} + \text{lysolecithin}$$

$$\text{enzyme–Ca} \rightleftarrows \text{enzyme} + \text{Ca}$$

It may be noted in eq A-16, that the inhibition constant for butyramide is multiplied by $(k_5 + k_7)/k_5$. The value of this apparent K_i was derived from Figure 11A (Table I K_{iBN}'). In eq A-17 and A-18 both K_i' and the true K_i appear. An approximate value for K_i can be calculated according to eq A-18 from the inhibition data obtained in the presence of butyramide and lysobutyryllecithin (Figure 11B). The calculated value is found in Table I. This value must be consid-

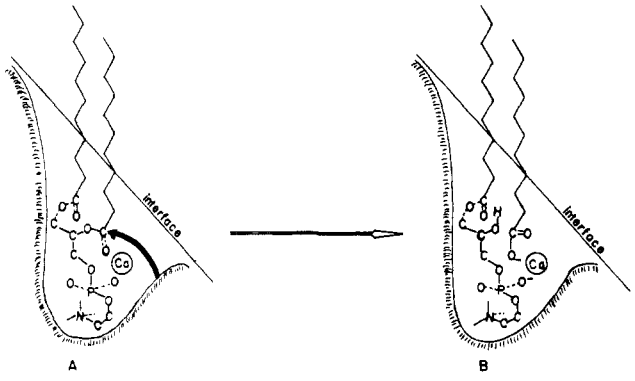


FIGURE 13: Schematic representation of the binding of lecithin to phospholipase A_2. (A) Binding of substrate showing the proposed role of Ca^{2+} and the primary importance of the polar part of the molecule in binding. The arrow indicates nucleophilic attack on the carbonyl carbon. (B) Binding of products to phospholipase A_2. The fatty acid and then the lysolecithin can leave if the substrate is monomeric, but in the case of aggregate substrates the enzyme dissociates from the interface.

ered only approximate since the calculation involves the use of several constants. However, a combination of K_i and K_i' allows an approximation of $(k_5 + k_7)/k_5$. The value of this ratio is found in Table I. The point of this calculation is to show that the rate of release of butyric acid and lysobutyryllecithin must be approximately the same.

Wells (1971a) was unable to obtain any evidence for an acyl-enzyme intermediate in this reaction. The data obtained here would be consistent with such an observation. If an acyl-enzyme intermediate were part of the catalytic mechanism of this enzyme, then deacylation would have to precede release of lysobutyryllecithin. Deacylation might then be very rapid and the presence of the lysobutyryllecithin in the active site might exclude the entrance of other nucleophiles. An equally plausible mechanism would involve a concerted hydrolytic step without the formation of an acyl-enzyme intermediate. It would be difficult to distinguish between these mechanisms if the rate of deacylation were much faster than the rate of acylation.

It may be noted that the observations using this monomeric substrate give results which are also compatible with a plausible mechanism for the hydrolysis of aggregated substrates. When the reaction occurs at an interface, it is reasonable to assume that both products will remain in the interface, and so it would not be possible to have a mechanism which requires one product to dissociate from the enzyme before the the reaction could be completed. For example, a mechanism requiring the formation of an acyl-enzyme intermediate, which could only be deacylated after the lysolecithin had left the enzyme, would be improbable and is not consistent with the data presented here. However, a mechanism which allows for hydrolysis without the necessity for the lysolecithin to leave the enzyme before the reaction is completed would be reasonable and consistent with the data presented in this paper.

Such a mechanism could be envisaged as follows (Figure 13). It is proposed that the binding of the substrate to the enzyme involves primarily the polar part of the molecule, the carbonyl oxygen of the ester to be hydrolyzed, and a few, but limited number, of the methylene carbons of the fatty acid chain. It is also proposed that Ca^{2+} is involved in binding the substrate through interaction with the negatively charged oxygen on the phosphorus and the carbonyl oxygen of the ester to be hydrolyzed (Figure 13A). This later interaction would produce a partial positive charge on the carbonyl carbon and promote nucleophilic attack. Hydrolysis occurs either through a concerted attack or an acylation–deacylation mechanism in which deacylation is more rapid than acylation. The products are then bound to the enzyme as depicted in Figure 13B. If the substrate is monomeric, the fatty acid and then the lysolecithin are released. If the substrate is aggregated, the enzyme dissociates from the interface, leaving both products behind.

Two other observations should be mentioned at this point. The pH–activity data, Figure 12, might be taken to indicate that an unprotonated histidine is involved in the catalysis. Further work on groups involved in catalysis is underway. The fact that glycerophosphorylcholine does not inhibit the enzyme stimulates some interesting speculation about the binding of substrate to the enzyme. Although the phosphorylcholine moiety must be present for the lecithin to act as a substrate, this result would suggest that the acyl group at C-1 is very important for binding to the enzyme. The fact that K_{DBL} is considerably lower than K_{iLBL} would tempt one to propose that the acyl group at C-2 is also very important for binding. However, the complex nature of K_{DBL} means that further work will be necessary to establish this point.

After this work was completed, de Haas *et al.* (1971) reported a kinetic study on short chain lecithins using the pancreatic phospholipase A_2. In contrast to the data reported in this paper, they studied hydrolysis under conditions where the substrate was present as micelles. The point of primary interest here is the evidence they present which shows that the addition of Ca^{2+} and lecithin to this phospholipase A_2 is random.[2]

One possible explanation for the different results is that, although these two enzymes catalyze the same reaction, they do so by different mechanisms. There is some evidence in support of this proposal. The pH optimum which they report for the pancreatic enzyme is near 6.0, whereas the data reported here show an optimum near 8.0, and no activity at pH 6.0. It is possible, however, that their lower pH optimum may be due to a surface pH effect at the interface of the lecithin micelle. Another difference is the apparent poorly defined activity of the pancreatic enzyme toward monomeric substrates.

If, however, the mechanisms are the same, there are still several possible explanations for the difference in results. All these possibilities center on the peculiar properties of micellar substrates, and point out again the difficulties of making unequivolcal statements about kinetic studies obtained on micellar substrates. Since the substrate is part of an interface, it is probably correct to think of the enzyme adsorbing to the interface and then finding a substrate molecular to hydrolyze. There is no *a priori* reason to assume that the enzyme must leave the interface before attacking another substrate molecule, and this may account for the rate enhancements observed when micellar substrates are compared to monomeric substrates (Figures 1 and 2 and de Haas *et al.*, 1971). The effect of such a mechanism is to decrease the slopes of the lines in $1/v$ *vs.* $1/S$ plots, but the initial velocity pattern is not altered.[3] By itself then this phenomena could not explain the difference in results.

[2] The initial velocity pattern reported by de Haas *et al.* (1971) is not sufficient evidence to prove a random mechanism (Cleland, 1970, p 8).

[3] M. A. Wells, unpublished calculations.

If the Ca^{2+} must be attached to the enzyme before it can interact with the lecithin, there are at least two ways in which the properties of the interface could alter the initial velocity patterns and make the addition appear random. The enzyme–Ca^{2+} complex might bind to the substrate molecule in the interface, catalyze a reaction, but the enzyme will leave the interface as free enzyme. The Ca^{2+} would be retained in the interface due to the high affinity of carboxylate groups in interfaces for Ca^{2+} (Gaines, 1966). Such a mechanism would alter the initial velocity patterns from that observed here toward that observed by de Haas *et al.* (1971).

Another possible mechanism involves nonproductive binding of the enzyme to the micelle. The free enzyme may be attracted to the interface as it would be to any interface, but the interaction would be nonproductive in the sense that there would be no specificity involved in the interaction. While still in the interface, the enzyme could bind calcium and then form a productive complex with a substrate molecule in the interface. Such a mechanism would predict random addition of calcium and lecithin, but in fact, only an ordered addition leads to productive binding. Partial support for this type of interaction was obtained in this laboratory.[4] The phospholipase will absorb as a soluble film to a hexane–water interface. However, if lecithin is spread at the hexane–water interface, the enzyme absorbs as an insoluble film, whether calcium is present or not. This would indicate that the enzyme can interact with the lipid at an interface whether calcium is present or not.

If both enzymes are capable of proceeding by a random mechanism, then the expression of the randomness depends on the relative rates of release of Ca^{2+} and products from the enzyme, or enzyme from the product in the case of micellar substrates. In the case of monomeric substrates the release of products may be so much faster than the release of Ca^{2+} that the mechanism appears ordered. In the case of the micellar substrate the release of enzyme from the interface may be slow and comparable to the rate of Ca^{2+} release. In such a case the random nature of the mechanism would be expressed.

The reason for belaboring this point, is that in order to understand the mechanism of phospholipase A_2 it is obviously important to know whether Ca^{2+} must be bound to the enzyme before lecithin can be bound, or whether binding can indeed be random. It is not sufficient to show that the enzyme can bind to a micelle in the absence of Ca^{2+}, since this may be nonproductive binding. In order to resolve this question we are now measuring the release of Ca^{2+} by rapid kinetic methods. Further work on both enzymes must be done before the similarities and differences are well enough defined to make detailed comparisons meaningful.

While the data presented in this paper are reasonably consistent with the proposed mechanism there are some points which need to be clarified. First, it must be established whether the acyl group is released as the carboxylate or carboxyl form in order to substantiate the use of butyramide as a product analog. Secondly, the rate of calcium release compared to the rate of product release from the enzyme must be determined in order to show, conclusively whether the mechanism is ordered, and not a consequence of a slow release of calcium from enzyme. Thirdly, more extensive work is needed on the characterization of the interaction of the enzyme with interfacial substrates in order to determine whether or not the apparent mechanism is changed when these substrates are used as compared to monomeric substrates.

[4] J. Mingins and M. A. Wells, unpublished observations.

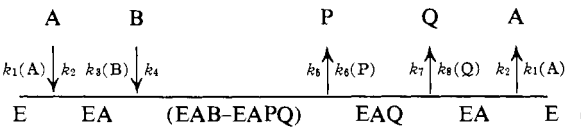
Acknowledgments

The expert technical assistance of Mrs. Norma Hewlett is gratefully acknowledged. I thank Mr. Ronald Misiorowski for stimulating discussions.

Appendix

Rate Equations for Some Bi-Ter Reactions

I. Ordered bi-ter.



(A-1)

Rate equation (King and Altman, 1956).

$$v = \frac{[k_1k_3k_5k_7(\mathrm{AB}) - k_1k_4k_6k_8(\mathrm{APQ})]E_t}{[k_2k_7(k_4 + k_5) + k_1k_7(k_4 + k_5)(\mathrm{A}) + k_1k_3(k_5 + k_7)(\mathrm{AB}) + k_2k_4k_6(\mathrm{P}) + k_1k_4k_6(\mathrm{AP}) + k_1k_8(k_4 + k_5)(\mathrm{AQ}) + k_1k_6k_8(\mathrm{APQ}) + k_1k_3k_6(\mathrm{ABP})]} \quad \text{(A-2)}$$

which transforms into (Cleland, 1963a).

$$v = \frac{V_1V_2\left[(\mathrm{AB}) - \frac{(\mathrm{APQ})}{K_{eq}}\right]}{K_{ia}K_bV_2 + K_bV_2(\mathrm{A}) + V_2(\mathrm{AB}) + K_{ia}K_q\frac{V_1}{K_{eq}}(\mathrm{P}) + K_p\frac{V_1}{K_{eq}}(\mathrm{AQ}) + K_q\frac{V_1}{K_{eq}}(\mathrm{AP}) + \frac{V_1}{K_{eq}}(\mathrm{APQ}) + \frac{V_2}{K_{ip}}(\mathrm{ABP})} \quad \text{(A-3)}$$

where

$$K_{ia} = \frac{k_2}{k_1} \qquad K_b = \frac{k_1k_7(k_4 + k_5)}{k_1k_3(k_5 + k_7)}$$

$$K_q = \frac{k_4}{k_8} \qquad K_p = \frac{k_4 + k_5}{k_6}$$

$$K_{ip} = \frac{k_5 + k_7}{k_6} \qquad K_{iq} = \frac{k_7}{k_8}$$

$$K_{eq} = \frac{K_pK_{iq}}{K_b}$$

Initial Velocity Patterns

A. (P) = (Q) = 0.

A as variable substrate

$$\frac{1}{v} = \frac{K_{ia}}{V_1}\left[\frac{K_b}{(\mathrm{B})}\right]\frac{1}{(\mathrm{A})} + \frac{1}{V_1}\left[1 + \frac{K_b}{(\mathrm{B})}\right] \quad \text{(A-4)}$$

B as variable substrate

$$\frac{1}{v} = \frac{K_b}{V_1}\left[1 + \frac{K_{ia}}{(A)}\right]\frac{1}{(B)} + \frac{1}{V_1} \quad \text{(A-5)}$$

B. PRODUCT INHIBITION BY P, (Q) = 0

A as variable substrate

$$\frac{1}{v} = \frac{K_{ia}}{V_1}\left\{\frac{K_b}{(B)}\left[1 + \frac{(P)}{\frac{K_pK_{iq}}{K_q}}\right]\right\}\frac{1}{(A)} + \frac{1}{V_1}\left[1 + \frac{K_b}{(B)}\right]\left\{1 + \frac{\left[\frac{(P)}{1 + \frac{K_b}{(B)}}\right]}{\left[\frac{1}{K_{ip}} + \frac{K_qK_b}{K_pK_{iq}(B)}\right]}\right\} \quad \text{(A-6)}$$

B as variable substrate

$$\frac{1}{v} = \frac{K_b}{V_1}\left[1 + \frac{K_{ia}}{(A)}\right]\left[1 + \frac{(P)}{\frac{K_pK_{iq}}{K_q}}\right]\frac{1}{(B)} + \frac{1}{V_1}\left[1 + \frac{(P)}{K_{ip}}\right] \quad \text{(A-7)}$$

C. PRODUCT INHIBITION BY Q, (P) = 0.

A as variable substrate

$$\frac{1}{v} = \frac{K_{ia}}{V_1}\left[\frac{K_b}{(B)}\right]\frac{1}{(A)} + \frac{1}{V_1}\left[1 + \frac{K_b}{(B)}\right]\left\{1 + \frac{(Q)}{K_{iq}\left[1 + \frac{(B)}{K_b}\right]}\right\} \quad \text{(A-8)}$$

B as variable substrate

$$\frac{1}{v} = \frac{K_b}{V_1}\left[1 + \frac{K_{ia}}{(A)}\right]\left\{1 + \frac{(Q)}{K_{iq}\left[1 + \frac{K_{ia}}{(A)}\right]}\right\}\frac{1}{(B)} + \frac{1}{V_1} \quad \text{(A-9)}$$

D. DEAD-END INHIBITORS. These equations were derived by the method of Cleland (1963b). K_i is the inhibition constant.

1. E + I, (P) = [Q] = 0

A as variable substrate

$$\frac{1}{v} = \frac{K_{ia}}{V_1}\frac{K_b}{(B)}\left[1 + \frac{(I)}{K_i}\right]\frac{1}{(A)} + \frac{1}{V_1}\left[1 + \frac{K_b}{(B)}\right] \quad \text{(A-10)}$$

B as variable substrate

$$\frac{1}{v} = \frac{K_b}{V_1}\left[1 + \frac{K_{ia}}{(A)}\right]\left\{1 + \frac{(I)}{K_i\left[1 + \frac{(A)}{K_{ia}}\right]}\right\}\frac{1}{(B)} + \frac{1}{V_1} \quad \text{(A-11)}$$

2. EA + I, (P) = (Q) = 0

A as variable substrate

$$\frac{1}{v} = \frac{K_{ia}}{V_1}\left[\frac{K_b}{(B)}\right]\frac{1}{(A)} + \frac{1}{V_1}\left\{1 + \frac{K_b}{(B)}\left[1 + \frac{(I)}{K_i}\right]\right\} \quad \text{(A-12)}$$

B as variable substrate

$$\frac{1}{v} = \frac{K_b}{V_1}\left[1 + \frac{K_{ia}}{(A)}\right]\left\{1 + \frac{(I)}{K_i\left[1 + \frac{K_{ia}}{(A)}\right]}\right\}\frac{1}{(B)} + \frac{1}{V_1} \quad \text{(A-13)}$$

3. EAQ + I, (P) = (Q) = 0

A as variable substrate

$$\frac{1}{v} = \frac{K_{ia}}{V_1}\left[\frac{K_b}{(B)}\right]\frac{1}{(A)} + \frac{1}{V_1}\left[1 + \frac{K_b}{(B)}\right]\left\{1 + \frac{I}{K_i'\left[1 + \frac{K_b}{(B)}\right]}\right\} \quad \text{(A-14)}$$

B as variable substrate

$$\frac{1}{v} = \frac{K_b}{V_1}\left[1 + \frac{K_{ia}}{(A)}\right]\frac{1}{(B)} + \frac{1}{V_1}\left[1 + \frac{(I)}{K_i'}\right] \quad \text{(A-15)}$$

where

$$K_i' = K_i\frac{k_5 + k_7}{k_5} \quad \text{(A-16)}$$

E. EAQ + I IN THE PRESENCE OF Q, (P) = 0.

A as variable substrate

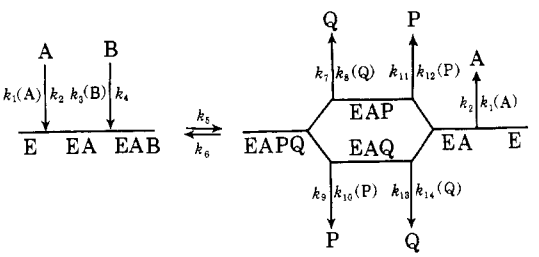
$$\frac{1}{v} = \frac{K_{ia}}{V_1}\left[\frac{K_b}{(B)}\right]\frac{1}{(A)} + \frac{1}{V_1}\left[1 + \frac{K_b}{(B)}\right]\left\{1 + \frac{I}{K_i'\left[1 + \frac{K_b}{(B)}\right]} + \frac{(Q)\left[1 + \frac{(I)}{K_i}\right]}{K_{iq}\left[1 + \frac{(B)}{K_b}\right]}\right\} \quad \text{(A-17)}$$

B as variable substrate

$$\frac{1}{v} = \frac{K_b}{V_1}\left[1 + \frac{K_{ia}}{(A)}\right]\left\{1 + \frac{(Q)\left[1 + \frac{(I)}{K_i}\right]}{K_{iq}\left[1 + \frac{K_{ia}}{(A)}\right]}\right\}\frac{1}{(B)} + \frac{1}{V_1}\left[1 + \frac{(I)}{K_i'}\right] \quad \text{(A-18)}$$

K_i' is defined as in eq A-16 and K_i is the true inhibition constant.

II. Rapid-Equilibrium-Ordered Addition of Reactants, Rapid Equilibrium Random Release of Products with the Rate-Determining Step the Interconversion of Two Central Complexes.



(A-19)

rate equation

$$v = \frac{V_1V_2\left[(AB) - \frac{(APQ)}{K_{eq}}\right]}{K_{ia}K_bV_2 + K_bV_2(A) + V_2(AB) + \frac{V_1}{K_{eq}}(APQ) + \frac{V_1}{K_{eq}}K_q(AP) + \frac{V_1}{K_{eq}}\frac{K_{ip}K_q}{K_{ip}}(AQ)} \quad \text{(A-20)}$$

where $K_{ia} = k_2/k_1$, $K_q = k_7/k_8$, $K_b = k_4/k_3$, $K_{ip} = k_{11}/k_{12}$, $K_{iq} = k_{13}/k_{14}$, and $K_{eq} = K_{ip}K_q/K_b$. Initial velocity patterns.

A. (P) = (Q) = 0. These equations are identical with eq A-4 and A-5.

B. Product inhibition by P, (Q) = 0. These equations are identical with eq A-8 and A-9 except that K_{ip} replaces K_{iq}.

C. Product inhibition by Q, (P) = 0. These equations are identical with eq A-8 and A-9.

D. Dead-end inhibitors. (1) E + I, (P) = (Q) = 0. These equations are identical with eq A-10 and A-11. (2) EA + I, (P) = (Q) = 0. These equations are identical with eq A-12 and A-13.

(3) EAQ + I, (P) = (Q) = 0. This mechanism predicts no inhibition in the absence of Q.

(4) EAQ + I in the presence of Q, (P) = 0.

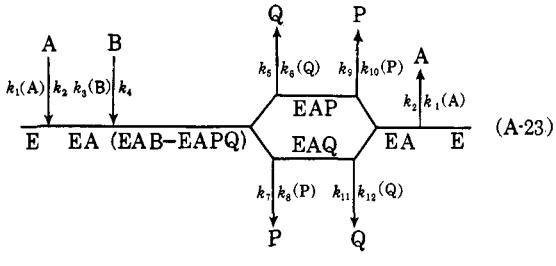
A as variable substrate

$$\frac{1}{v} = \frac{K_{ia}K_b}{V_1}\frac{1}{(B)(A)} + \frac{1}{V_i}\left(\left[1 + \frac{K_b}{(B)}\right]\left\{1 + \frac{(Q)}{K_{iq}}\left[\frac{1 + \frac{(I)}{K_i}}{1 + \frac{(B)}{K_b}}\right]\right\}\right) \quad \text{(A-21)}$$

B as variable substrate

$$\frac{1}{v} = \frac{K_b}{V_1}\left(\left[1 + \frac{K_{ia}}{(A)}\right]\left\{1 + \frac{(Q)}{K_{iq}}\left[\frac{1 + \frac{(I)}{K_i}}{1 + \frac{K_{ia}}{(A)}}\right]\right\}\right) + \frac{1}{V_1} \quad \text{(A-22)}$$

III. Ordered Addition of Reactants with Random Release of Products.



(A-23)

The rate equation for this mechanism is complex with terms containing ABP, ABQ, AP^2Q, and APQ^2 in the numerator, and terms in AP^2, AQ^2, AP^2Q, and APQ^2, etc., in the denominator. In addition the denominator contains 41 terms. This equation has not been placed in coefficient form, but the presence of P, Q, AP, AQ, ABP, and ABQ terms in the denominator and the rules for predicting product inhibition given by Cleland (1963c) show that both products will be noncompetitive and nonlinear inhibitors with respect to both substrates.

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