Privett, O. S., Blank, M. L., and Romanus, O. (1963), *J. Lipid Res. 4*, 260.
Schlossberger, H. (1938), *in* Handbuch der Pharmakologie, Ergänzungswerk, Vol. 5, Heubner, W., and Schüller, J., Ed., Berlin, Springer-Verlag, p 1.
Schmid, H. H. O., Jones, L. L., and Mangold, H. K. (1967), *J. Lipid Res. 8*, 692.
Shriner, R. L., and Adams, R. (1925), *J. Amer. Chem. Soc. 47*, 2727.
Siakotos, A. N., and Rouser, G. (1965), *J. Amer. Oil Chem. Soc. 42*, 913.
Slotta, K. (1966), *Monatsh. Chem. 97*, 1723.
Sousa, J. A., and Bluhm, A. L. (1960), *J. Org. Chem. 25*, 108.
Spener, F. (1973), *Chem. Phys. Lipids 11*, 229.
Spener, F., and Mangold, H. K. (1973), *Chem. Phys. Lipids 11*, 215.
Spener, F., Staba, E. J., and Mangold, H. K. (1974), *Chem. Phys. Lipids* (in press).
Stein, R. A., and Nicolaides, N. (1962), *J. Lipid Res. 3*, 476.
Stoffel, W., Chu, F., and Ahrens, Jr., E. H. (1959), *Anal. Chem. 31*, 307.
Stumpf, P. K. (1963), *Proc. Int. Congr. Biochem., 5th, 1961, 7*, 80.
Sumerwell, W. N. (1957), *J. Amer. Chem. Soc. 79*, 3411.
Vandyke, R. H., and Adams, R. (1926), *J. Amer. Chem. Soc. 48*, 2393.
Willner, D. (1965), *Chem. Ind. (London)* 1839.
Zeman, I., and Pokorny, J. (1963), *J. Chromatogr. 10*, 15.

The Mechanism of Interfacial Activation of Phospholipase A_2†

Michael A. Wells

ABSTRACT: The activating effect of substrate aggregation on the activity of *Crotalus adamanteus* phospholipase A_2 was investigated. Kinetic analyses were carried out using dibutyryl-, dihexanoyl-, and dioctanoyllecithin at 45° both below and above the critical micelle concentration. The major source of the enhanced rate of hydrolysis of the aggregated substrates lies in a much lower entropy of activation. It is suggested that the origin of this entropy difference probably arises from the fact that in the aggregated state, only the reactive end of the substrate molecule is presented to the enzyme during collisions. An additional factor might arise from conformational constraints placed on the glycerophosphoryl group of the substrate in the aggregate state. Data are presented which suggested that a negative charge is present at the surface of the aggregate which markedly influences the pH dependence of the reaction and the apparent kinetic mechanism. These effects are sensitive to the addition of salts such as KCl or $MgCl_2$, which influence the surface charge. The validity of the steady-state kinetic analysis of aggregate substrates was investigated and conditions were defined under which meaningful kinetic data can be collected. The meaning of K_m and V_m for these systems has also been explored. No evidence for hydrophobic interaction of the enzyme with the interface could be found, and it is suggested that the activating effect of the interface is not related to irreversible adsorption to the interface.

It is well known that the physical properties of the substrate can markedly influence the activity of phospholipase A_2 (EC 3.1.1.4) (see Wells (1972) for references to earlier studies). Recent studies (deHaas *et al.*, 1971; Wells, 1972; Pieterson, 1973) have shown that the aggregated form of short-chain lecithins is a considerably better substrate than the monomeric form.

The origin of this activation by substrate aggregation is unknown. Verger *et al.* (1973), studying pancreatic phospholipase A_2, have proposed that the enzyme penetrates between the lecithin molecules in the interface, but it is not clear how this penetration could lead to enhanced activity. Pieterson (1973) has proposed an "anchoring" site on the enzyme which somehow can account for interfacial activation. At present the nature of this "anchoring" site remains vague, although in a kinetic sense it can account for enhanced rates in the presence of aggregated substrates.

It has been suggested that substrate aggregation might alter the apparent kinetic mechanism of the enzyme (Wells, 1972). To date the only detailed kinetic analysis of monomeric substrates has been performed using the *Crotalus adamanteus* (Eastern Diamondback Rattlesnake) enzyme (Wells, 1972), while the only detailed kinetic analysis of aggregated substrates has been performed using the pancreatic enzyme (deHaas *et al.*, 1971).

The purpose of this study was to explore possible mechanisms of the interfacial activation of phospholipase A_2 and to compare the apparent kinetic mechanism of the hydrolysis of monomeric and aggregated substrates using *C. adamanteus* phospholipase A_2.

Materials and Methods

Materials. Enzyme purification and substrate (dibutyryl-, dihexanoyl-, and dioctanoyllecithins) preparation have been described previously (Wells and Hanahan, 1969; Wells, 1972; Misiorowski and Wells, 1973). Ammonium purpurate (murexide) was purchased from K and K Laboratories (Plainview, N. Y.), Phenol Red from J. T. Baker Co. (Phillipsburg, N. J.), 8-anilino-1-naphthalenesulfonic acid (ANS)[1] was purchased

† From the Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Arizona 85724. *Received November 30, 1973.* Supported by a grant from the National Science Foundation (GB-35527).

[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonic acid; cmc, critical micelle concentration.

from Eastman (Rochester, N. Y.), and other chemicals were reagent grade and used as received.

Methods. The enzyme assay was as described previously (Wells, 1972) with the following modifications for use with dioctanoyllecithin. This substrate does not form a clear solution in water below 40°; therefore the required amount of a methanolic solution of substrate was added to the titration vessel and solvent removed under N_2; 1 ml of water containing $CaCl_2$ and other additions as appropriate was added and the solution brought to 45°. After 5 min the pH was adjusted, a base line recorded, and the reaction initiated with 1.5 ng of enzyme in 10 μl of water. The reaction was followed for 5 min. Kinetic data were analyzed as described previously (Wells, 1972). Temperature studies using dibutyryllecithin were carried out at 5° intervals from 15 to 60°, while for dioctanoyllecithin at 4° intervals between 45 and 65°. Activation energies were calculated in the usual way (Frost and Pearson, 1961).

The surface charge of lecithin aggregates was investigated either by measurement of the rate of OH^- catalyzed hydrolysis or by surface pH measurements. The rate of hydroxide ion catalyzed hydrolysis of lecithins was determined at 45° using either 20 mM dibutyryl- or dioctanoyllecithin under N_2 in the pH-Stat. The rate was determined from the amount of base required to maintain the selected pH. By analogy with hydrolytic studies on monolayers (Davies and Rideal, 1963), the difference in the rate of hydrolysis of dibutyryl- (DBL) (monomeric) and dioctanoyl- (DOL) (aggregate) lecithins can be related to the surface potential of the aggregated substrate by

$$V_{DOL}/V_{DBL} = e^{-Z_2\epsilon\psi/kT} \quad (1)$$

where V_{DOL} = rate of hydrolysis of dioctanoyllecithin, V_{DBL} = rate of hydrolysis of dibutyryllecithin, Z_2 = charge of attacking species (OH^-), ϵ = electronic charge, ψ = surface potential, k = Boltzmann constant, and T = absolute temperature. At 45° and with ψ expressed in millivolts this reduced to

$$\psi = 27.41 \ln (V_{DOL}/V_{DBL}) \quad (2)$$

Surface pH was measured using Phenol Red as an indicator; 4 ml of a 2×10^{-5} M solution of Phenol Red was placed in the pH-Stat titration vessel. The solution was pumped by a peristaltic pump through a flow cell (1-cm path length) in a water jacketed cell holder in a Cary Model 15 and then back to the titration vessel. Both the titration vessel and the cell holder were maintained at 45.0° with a circulating water pump. The flow rate was approximately 50 ml/hr, and the titration vessel was maintained in a N_2 atmosphere. After temperature equilibrium (30 min), the pH was adjusted using 0.01 N NaOH. When the pH had remained constant for 5 min, the spectrum was recorded from 650 to 400 nm. The solution was adjusted to a new pH and the spectrum recorded again. This was repeated until there was no further change in the spectrum with additional NaOH. The absorbancy of the basic form of the indicator (λ_{max} 558 nM, $a_m = 4.59 \times 10^4$) was used to construct a standard curve.

The above procedure was then repeated using 20 mM dibutyryl- or dioctanoyllecithin and other additions as appropriate. The bulk pH (pH_b) was determined from the pH meter reading and the surface pH (pH_s) from the absorbancy of the indicator. The surface potential was calculated as (Davies and Rideal, 1963)

$$pH_s = pH_b + \epsilon\psi/2.3kT \quad (3)$$

At 45° and with ψ expressed in millivolts this reduces to

$$\psi = 63.04(pH_s - pH_b) \quad (4)$$

The possible binding of calcium to lecithins was studied using murexide. The experiment was conducted essentially as described for the surface pH studies using 1×10^{-4} M murexide and scanning from 650 to 400 nm. The formation of the calcium–murexide complex was followed by the increase in absorbance at 600 nm. The data are reported as

$$_s[Ca\text{–}M]/_b[Ca\text{–}M] \quad (5)$$

where $_s[Ca\text{–}M] = \Delta A_{600}$ for a given addition of calcium in the presence of an aggegate, and $_b[Ca\text{–}M] = \Delta A_{600}$ for the same addition of calcium in the absence of an aggregate, all other conditions being the same.

The effect of phospholipase A_2 on the fluorescence of ANS was measured in a Perkin-Elmer fluorescence spectrophotometer, Model MPF-2A. Excitation and emission slits were set at 10 nm. Both excitation and emission spectra were recorded. Measurements were made at pH 8.0 in 0.05 M Tris buffer. Protein concentration ranged from 0.05 to 5 mg/ml, and the concentration of ANS ranged from 1×10^{-6} to 1×10^{-4} M.

Critical micelle concentration (cmc) was measured by the increase in fluorescence of ANS in the presence of a lipid aggregate. ANS (1×10^{-4} M) was mixed with different concentrations of lipid. The fluorescence emission at 495 mM (activation at 375 nM) was measured. The lipid concentration was varied from below the critical micelle concentration (no enhancement of ANS fluorescence) up to a concentration well above the critical micelle concentration. A plot of relative fluorescence *vs.* log c gave two linear portions, one of zero slope (below the cmc) and one of positive slope (above the cmc). The point of intersection of the two lines was taken as the cmc.

Fatty Acid Analyses. In some experiments mixtures of lecithins with different fatty acyl chains were incubated together with the enzyme. In order to analyze the released fatty acids without loss of the short chain fatty acids, the following procedure was used. The enzymatic reaction was stopped by addition of 0.1 M EDTA (pH 7.0). The reaction mixture adjusted to pH 7.0 and lyophilized. The residue was extracted with CS_2 made 1 N with HCl (gas) and an aliquot immediately analyzed for fatty acids. Free acids were analyzed either on (1) Porapak Q 50–80 mesh (Waters Assoc. Inc., Framingham, Mass.) in a 0.2 mM × 6 ft glass column at 180° (flash evaporator 200°, detector 265°, carrier gas helium 70 ml/min), retention time of butyric acid was 8 min; or on (2) SE-30 (2.25% on Chromosorb W AW-DMCS, Applied Science Lab, State College, Pa.) in a 0.25 in. × 4 ft stainless steel column at 75° (flash evaporator 80°, detector 200°, carrier gas helium 70 ml/min), retention time of butyric acid 1 min, octanoic acid 8 min. A Hewlett-Packard Model 402 gas chromatograph with flame detector and a Model 3370A integrator were used.

Sedimentation equilibrium studies of the molecular weight of dioctanoyllecithin aggregates were conducted at 45° and 10,000 rpm in the Beckman Model E analytical ultracentrifuge. An initial concentration of 5.6 mg/ml was used. Density measurements at 45.0° were made in a 25-ml pycnometer. The accuracy of the density measurements was ±0.0002 g/ml.

Results

Properties of Lecithin Aggregates. Three substrates were used in these studies: dibutyryl-, dihexanoyl-, and dioctanoyl-

lecithins. The first two form clear solutions in water at room temperature, whereas the latter does not. However, above 40° dioctanoyllecithin does form a clear solution and therefore all studies were conducted at 45°. The clearing of the dioctanoyllecithin solution probably represents a phase transition. The nature of the aggregate state below the phase transition is unknown (deHaas *et al.*, 1971). At 45° the following critical micelle concentration was determined: dibutyryllecithin in water, 80 ± 5 mM; dihexanoyllecithin in water, 9.8 ± 0.1 mM [at 37° Pieterson (1973) reports 9.5 mM, and Roholt and Schlamowitz (1961) report 10.4–11 mM at 20°]; dihexanoyllecithin in 2 M KCl, 3.0 ± 0.2 mM [at 37° Pieterson (1973) reports 3.7 mM in 2 M NaCl]; dioctanoyllecithin in water 0.17 ± 0.02 mM [at 37° Pieterson (1973) reports 0.19 mM].[2]

The cmc's determined by the method used here as well as the dye solubilization method used by Pieterson (1973) may be influenced by the presence of the dye. However, the rather close agreement of both methods for the cmc of dihexanoyllecithin with the results obtained by Roholt and Schlamowitz (1961) using diffusion and refractive index measurements suggest that the values may be substantially correct.

An attempt was made to measure the molecular weight of the dioctanoyllecithin aggregate at 45° by sedimentation equilibrium analysis. The value of $M(1 - \bar{v}\rho)$ was found to be 10,500; however, the density of the lecithin solution was so close to that of water that an accurate estimate of $\bar{v}$ could not be made. Within the accuracy of the pycnometric measurements a minimum molecular weight of 1×10^6 is estimated.

Validity of Kinetic Measurements on Aggregated Substrates. There are no theoretical objections to kinetic studies carried out with monomeric substrates; however, there are potential problems in the analysis of kinetic data obtained with aggregate substrates. In addition to previously raised questions (Wells, 1972) one must demonstrate that steady-state kinetic analysis is justified and define the meaning of K_m and V_m. To date this does not appear to have been done, although Verger *et al.* (1973) have considered a theoretical model for hydrolysis at an interface.

Above the critical micelle concentration there are two substrate species present: monomers and aggregates, each characterized by its own apparent K_m and V_m. Following Dixon and Webb (1964) we define the total hydrolysis of substrate (V_t) as

$$V_t = \frac{V_m^{mon}([Mon]/K_m^{mon}) + V_m^{agg}([Agg]/K_m^{agg})}{1 + ([Mon]/K_m^{mon}) + ([Agg]/K_m^{agg})} \quad (6)$$

where [Agg] and K_m^{agg} refer to the concentration of monomers present as aggregates and do not reflect the true concentration of the aggregate substrate.

Below the critical micelle concentration, [Agg] = 0, and eq 6 reduces to a simple Michaelis equation. However, above the critical micelle concentration both monomers and aggregates are present. If we assume that the phase separation model for micelle formation is applicable, then the concentration of monomers is held constant at the cmc and eq 6 can be written as

$$V_t = \frac{V_m^{mon}([cmc]/K_m^{mon})}{1 + ([cmc]/K_m^{mon}) + ([Agg]/K_m^{agg})} + \frac{V_m^{agg}([Agg]/K_m^{agg})}{1 + ([cmc]/K_m^{mon}) + ([Agg]/K_m^{agg})} \quad (7)$$

As the substrate concentration increases to infinity, it is apparent that $V_t = V_m^{agg}$. If $V_m^{agg} \gg V_{cmc}^{mon}$, as is the case, then a plot of $1/V_t$ *vs.* 1/[Agg] should yield V_m^{agg} (deHaas *et al.*, 1971). However the K_m^{agg} determined from such a plot will not be the true K_m^{agg}, but will be

$$(K_m^{agg})_{app} = (K_m^{agg})_{true}\left[1 + \frac{[cmc]}{K_m^{mon}}\right] \quad (8)$$

In other words, the monomer acts as a competitive inhibitor of the hydrolysis of the aggregate.

If the mass action model of micelle formation is applicable, then eq 7 and 8 are not appropriate since the concentration of monomer will increase continuously above the cmc and eq 6 would hold.

The applicability of the above analysis to the phospholipase A_2 catalyzed hydrolysis of phosphatidylcholine micelles was tested in two ways. The first approach was to determine if the monomer was a competitive inhibitor of the hydrolysis of the aggregate. Since the monomer concentration is fixed at the cmc or increases slowly in an unknown manner above the cmc, this experiment could not be conducted with only one lecithin. However, eq 6 is not restricted to the monomer–aggregate pair of one lecithin, but applies to any monomer–aggregate pair. Therefore, one could use a monomer with a high cmc, dibutyryllecithin, and an aggregate with a low cmc, dioctanoyllecithin. As will be detailed below, the aggregate of dioctanoyllecithin is hydrolyzed some 10^4 times faster than the monomer of dibutyryllecithin; therefore, in this mixture, one would predict preferential hydrolysis of dioctanoyllecithin. However, if the dibutyryllecithin concentration is near its K_m one would also predict inhibition. Dibutyryllecithin was shown to be a linear competitive inhibitor of dioctanoyllecithin hydrolysis with a K_i = 40 mM which is the same as the K_m for hydrolysis of dibutyryllecithin. Table I provides data showing that only dioctanoyllecithin was hydrolyzed in this experiment. At Ca^{2+} concentration used (10^{-3} M) the enzyme will be saturated with calcium (Wells, 1972) and the reaction will be essentially a one-substrate reaction as is required by eq 6, and these data justify the analysis used to obtain eq 6.

The above analysis assumes that the mixture of dibutyryl- and dioctanoyllecithins is composed of predominately dibutyryllecithin monomers and dioctanoyllecithin micelles with a small amount of dioctanoyllecithin monomers. It is considered unlikely that significant amounts of dibutyryllecithin are present in the dioctanoyllecithin micelle for the following reasons: (1) the rate of hydrolysis of dibutyryllecithin micelles is about 0.25 the rate of hydrolysis of dioctanoyllecithin micelles, (2) the data in Table I shows that less than 0.05 μmol of butyric acid was produced during the reaction, (3) therefore it can be estimated that less than 1% of the dibutyryllecithin is present in a mixed micelle. These data also eliminate one possible mode of interfacial activation which supposes that the enzyme is bound to the interface in a conformation which is highly reactive toward monomers.

The second approach was to analyze the effect of substrate concentration on the velocity of the enzymatic reaction. Equation 7 predicts an abrupt change in slope of $1/v$ *vs.* $1/S$ plots at the cmc, and a linear plot of $1/v$ *vs.* 1/[Agg] where [Agg] = total concentration − cmc. Figure 1 shows a plot of S/v *vs.* S for the hydrolysis of dihexanoyllecithin at pH 8.0 and 45°. This type of plot was used in order to present all the data. Below the cmc there is a linear dependence of S/v on S, and above 25 mM there is also a linear portion; however, the region between 10 and 25 mM gives anomalous S/v *vs.* S plots.

[2] The nature of these micelles at 45° is unknown at the present time.

TABLE I: Fatty Acid Analysis of the Hydrolysis of Dioctanoyl- and Dibutyryllecithin Mixtures.[a]

Sample	Base Taken up at End Point (mequiv)	Relative Area on Gas Chromatograph	
		Octanoic	Butyric
1. 1 mM Dioctanoyllecithin	1.0[b]	1.0	*d*
2. 1 mM Dioctanoyllecithin 50 mM Dibutyryllecithin	1.05[c]	1.0	*d*
3. 50 mM Dibutyryllecithin	<0.01[c]	*d*	*d*
4. 1 mM Dioctanoyllecithin 50 mM Dibutyryllecithin	Not incubated	*d*	*d*
(a) 1 mequiv of octanoic acid 1 mequiv of butyric acid added before extraction	Not incubated	0.56	0.44
(b) 1 mequiv of octanoic acid 0.05 mequiv of butyric acid added before extraction	Not incubated	0.97	0.03

[a] Samples containing the indicated substrates were incubated at 45° and pH 8.0 with 0.1 μg of phospholipase A_2. The end point represents the point where base uptake was reduced to less than 5% of the initial rate. [b] 8 min. [c] 20 min. [d] None detectable.

Lineweaver–Burk plots of the monomer region (not shown) were linear and gave K_m = 4 mM, V_m = 6.9 μequiv min^{-1} mg^{-1}.

The data obtained between 25 and 100 mM substrate can be fitted to a linear Lineweaver–Burk plot by assuming a value of cmc between 9.5 and 10.0 mM. Higher or lower values for cmc lead to pronounced curvature of these plots. These data gave $(K_m)_{app}$ = 200 mM, $(K_m)_{True}$ = 58 mM (assuming cmc = 9.8 mM), and $V_m = 1 \times 10^4$ μequiv min^{-1} mg^{-1}. In this region there is no evidence that the concentration of the monomer differs significantly from the cmc. These results justify the use of the phase separation model in the derivation of eq 7 and 8 and the use of eq 8 for the calculation of V_m and K_m. It should be emphasized that these results do not prove that the phase separation model is applicable to a description of the micellization process, since a small change in monomer concentration above the cmc would not significantly affect the enzymatic rate.

The data obtained between 10 and 25 mM cannot be fitted to a linear Lineweaver–Burk plot by assuming any value for cmc. It is probable that this transition region represents the formation of larger aggregates and only above 25 mM is an aggregate of consistent size formed (Winsor, 1968). Alternatively, this region may be an expression of increasing monomer concentration as predicted by the mass action model. This effect may only be detected when the concentration of micelles is low. At present it is not possible to explain this anomalous region.

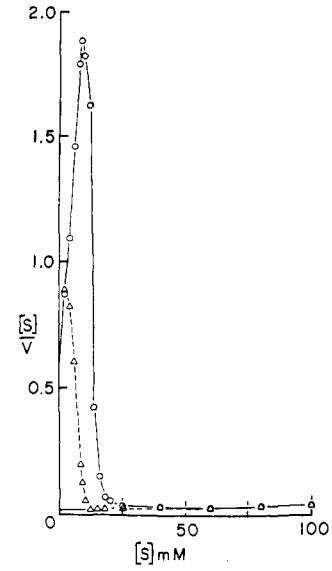

FIGURE 1: Effect of the concentration of dihexanoyllecithin on the rate of hydrolysis by phospholipase A_2. Reaction carried out at 45° and pH 8.0: (○) reaction in the presence of 1 mM Ca^{2+}; (△) reaction in the presence of 1 mM Ca^{2+} and 2 M KCl.

deHaas *et al.* (1971) reported that high salt concentrations enhance the activity of short-chain lecithins. As can be seen in Figure 1, 2 M KCl leads to apparent activation, but only at low (below 25 mM) substrate concentrations. A plot of $1/v$ *vs.* 1/[Agg] in the presence of 2 M KCl gives $V_m^{agg} = 5 \times 10^3$ μequiv min^{-1} mg^{-1} and $(K_m)_{app}^{agg}$ = 100 mM. In 2 M KCl the cmc is 3 mM and the true K_m^{agg} = 57 mM. The latter is not significantly different than found in the absence of KCl. Although data collected at low substrate concentration suggest that KCl activates, in fact V_m is lowered by the presence of KCl. The effect of KCl then is (1) to lower the cmc and thereby reduce the competition by the monomer and (2) to shift the anomalous region to lower substrate concentration. K_m and V_m for the monomer were not affected by 2 M KCl.

The results of these experiments do show that reasonable kinetic data can be obtained with aggregates by the use of sufficiently high concentrations of substrate and eq 8 to calculate kinetic parameters. However, careful attention must be paid to the exact conditions under which the experiments are conducted.

Kinetic Studies using Dioctanoyllecithin. In the previous section, we have outlined the manner in which a kinetic analysis of the hydrolysis of micelles should be approached. In order to minimize the effects of monomer inhibition dioctanoyllecithin (cmc ~0.2 mM) was chosen as the substrate for more detailed kinetic studies. The purpose of these studies was to determine whether the apparent kinetic mechanism for the hydrolysis of micelles was the same as previously reported for monomers (Wells, 1972), and, if not, to investigate the possible reasons for discrepancies.

The hydrolysis of dibutyryllecithin was shown to proceed by an ordered mechanism in which Ca^{2+} must be added to the enzyme before lecithin. Further, it was shown that the addition of Ca^{2+} was at thermodynamic equilibrium. The diagnostic features of this mechanism, as distinguished from other sequential or random mechanisms, are (1) the slope of $1/v$ *vs.* 1/[Ca] becomes zero at infinite lecithin concentration and (2) the intercepts at infinite lecithin concentration of $1/v$ *vs.* 1/[lecithin] plots are independent of the Ca^{2+} concentration used

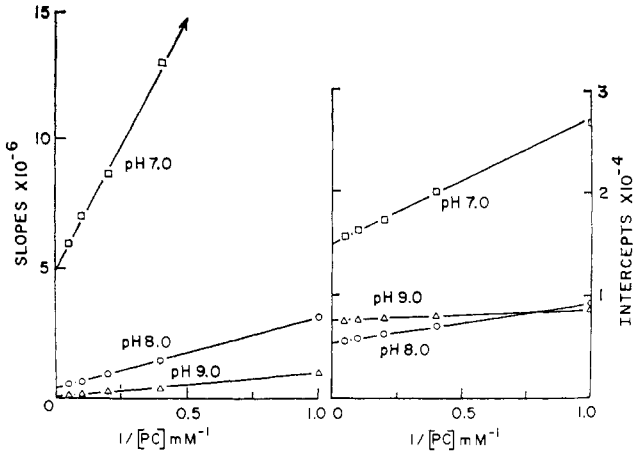

FIGURE 2: Effect of pH and dioctanoyllecithin concentration on the slopes and intercepts of double reciprocal plots of initial velocity as a function of Ca^{2+} concentration.

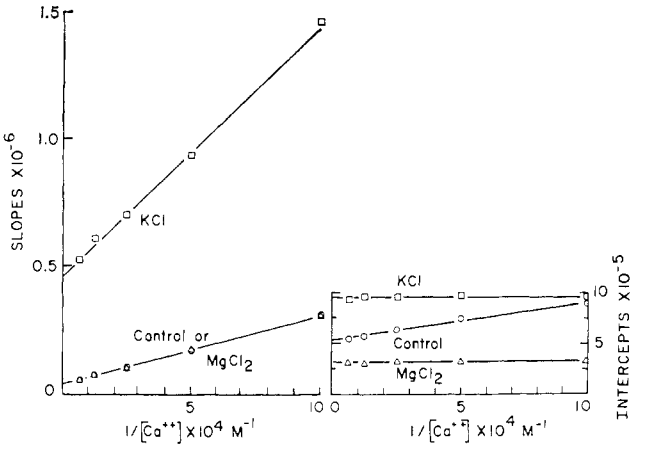

FIGURE 4: Effect of Ca^{2+} on the slopes and intercepts of double reciprocal plots of initial velocity as a function of dioctanoyllecithin at pH 8.0: control, no further addition; $MgCl_2$, addition of 0.01 M $MgCl_2$; KCl, addition of 0.15 M KCl.

in the experiment. Further this mechanism was observed from pH 7.0 to 9.0.

The data presented in Figures 2 and 3 summarize the kinetic data obtained with dioctanoyllecithin at pH 7.0, 8.0, and 9.0. Based on the criterion stated above, the hydrolysis at pH 9.0 follows an equilibrium ordered mechanism. However, as the pH is lowered, the data deviate strongly from that expected for an equilibrium ordered mechanism. Furthermore, V_m is lower at pH 9.0 than pH 8.0, in contrast to the results obtained with monomers.

When kinetic experiments were carried out in the presence of monovalent salts such as KCl or NaCl, the kinetic pattern was altered to that of an equilibrium ordered mechanism; however, the maximal velocity was lowered, as was observed for the hydrolysis of dihexanoyllecithin in 2 M KCl (see above). In addition, when the kinetics were studied in the presence of $MgCl_2$, the patterns were also altered to an equilibrium ordered mechanism, but the maximal velocity was increased. Figures 4 and 5 show the results obtained at pH 8.0 in the presence of either 0.15 M KCl or 0.01 M $MgCl_2$. Higher salt concentrations did not cause any additional effects. As shown in Table II, the effects of KCl and $MgCl_2$ on V_m were pH dependent. At all pH values studied (6.0–9.0) in the presence of KCl or $MgCl_2$, equilibrium ordered kinetics were observed.

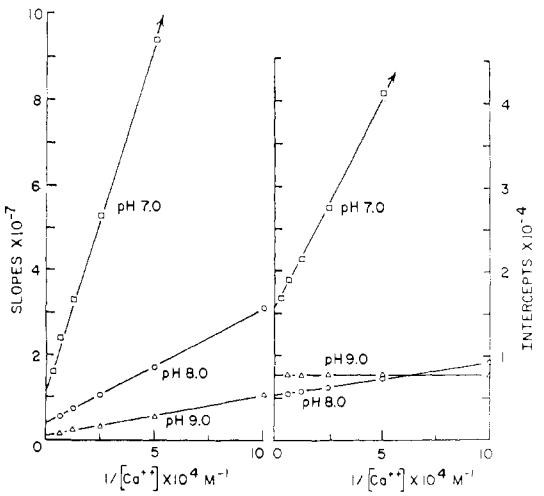

FIGURE 3: Effect of pH and Ca^{2+} concentration on the slopes and intercepts of double reciprocal plots of initial velocity as a function of dioctanoyllecithin concentration.

The stimulatory effect of Mg^{2+} was unexpected since Wells (1972, 1973) has shown that phospholipase A_2 does not bind Mg^{2+} and the reaction is absolutely specific for Ca^{2+}. In agreement with the latter observation there was no detectable enzymatic activity in the presence of Mg^{2+}, if Ca^{2+} were omitted from the reaction mixture.

By the criteria described above, the presence of either KCl or $MgCl_2$ alters the apparent kinetic mechanism to equilibrium ordered, but they do so by apparently different mechanisms. In the case of $MgCl_2$, V_m is increased, K_m for the substrate is raised slightly, and V_m/K_m, the apparent first-order rate constant, is increased. KCl, on the other hand, decreases V_m and V_m/K_m but increases K_m. These differences are summarized in Table III. In all cases K_{iCa}, which is the dissociation constant of the enzyme–Ca complex, is comparable to previously reported values (Wells, 1972, 1973). The primary difference between KCl and $MgCl_2$ seems to be an altered affinity of the enzyme for the substrate. (There was no significant effect of 0.15 M KCl or 0.01 M $MgCl_2$ on the cmc of dioctanoyllecithin.) The data shown in Figure 4 suggest that the anomalous kinetic effect of Ca^{2+}, observed in the absence of added ions, may result from a stimulatory effect of Ca^{2+} which is similar to the effect of Mg^{2+}.

Surface Charge Studies. Although the lecithin molecule is formally electrically neutral, it was considered possible that a small surface charge might be present which could affect the access of the negatively charged enzyme molecule to the substrate molecules in the interface. Such charge effects could

TABLE II: Effect of pH on V_m of the Phospholipase A_2 Hydrolysis of Dioctanoyllecithin.[a]

pH	V_m (μmol min^{-1}/mg^{-1})		
	None[b]	0.01 M $MgCl_2$[b]	0.15 M KCl[b]
6.0	1.5×10^3	1.6×10^3	1.5×10^3
7.0	6.7×10^3	9.8×10^3	4.4×10^3
8.0	18.9×10^3	34.5×10^3	11.1×10^3
9.0	13.5×10^3	30.5×10^3	8.5×10^3

[a] Data represent extrapolations of reciprocal plots to infinite Ca^{2+} and infinite lecithin. [b] Indicates components present in the assay other than $CaCl_2$ and dioctanoyllecithin.

TABLE III: Kinetic Constants[a] for the Hydrolysis of Dioctanoyllecithin at pH 8.0.

	None[b]	0.01 M $MgCl_2$[b]	0.15 M KCl[b]
V_m (μmol min^{-1} mg^{-1})	18.9×10^3	34.5×10^3	11.1×10^3
K_{DOL} (mM)	1.1	1.8	5.0
K_{iCa}	5.0×10^{-5} M	4.0×10^{-5} M	5.0×10^{-5} M
$\frac{V_m}{K_{DOL}}$ (mol/sec)	8.6×10^6	9.6×10^6	1.1×10^6

[a] See Wells (1972) for definition of kinetic constants. [b] Indicates components present in the assay other than $CaCl_2$ and dioctanoyllecithin.

reasonably be expected to be sensitive to the presence of KCl or $MgCl_2$ in the reaction medium.

One approach to studying surface charge is to measure surface pH. The results of these studies are shown in Figure 6 along with calculated surface potentials (eq 4). Although pH_s is only slightly different from pH_b, these results were quite reproducible. These data show the presence of a negative surface charge which is reduced by 0.15 M KCl and increased by 0.01 M $MgCl_2$ or 2×10^{-4} $CaCl_2$. If charge effects were important, it was difficult to understand why KCl should inhibit and $MgCl_2$ should activate the enzyme, since at pH 8.0 the enzyme would be negatively charged (Saito and Hanahan, 1962). As pointed out by Davies and Rideal (1963) the distance from the interface to the point which this potential measures is essentially unknown, since the exact location of the ionizable group of the indicator with respect to the polar head group of the lecithin within the surface cannot be determined.

If the aggregation of lecithin produces a negative surface charge, then hydrolysis of dioctanoyllecithin by OH^- might be inhibited relative to the hydrolysis of monomeric dibutyryllecithin which has no surface charge. Since the OH^- must penetrate the surface to reach the same functional group, *i.e.*, a carboxylic ester, as the enzyme, studies on OH^- hydrolysis might be more indicative of the local environment sensed by the enzyme than would surface pH measurements. Figure 7 shows the rate of hydrolysis of dibutyryl- and dioctanoyllecithin at 45° in H_2O, 0.15 M KCl, 2×10^{-4} M $CaCl_2$, and 0.01 M $MgCl_2$ as a function of a pH. As anticipated from the above discussion, the rate of hydrolysis of dioctanoyllecithin is much less than of dibutyryllecithin. Furthermore both Ca^{2+} and Mg^{2+} stimulate the hydrolysis of dioctanoyllecithin whereas KCl inhibits. There is no consistent effect of these ions on the hydrolysis of dibutyryllecithin. By use of eq 2, the surface potentials can be calculated. These values and values calculated from pH_s measurements are found in Table IV.

The effect of KCl and $MgCl_2$ on pH_s, V_{OH-}, and V_m depend on the concentration of the added salt. If there is a relation between V_m and surface potential then the dependence of the two on salt concentration should be correlated. Below pH 9.0 the rate of OH^- hydrolysis is too low to measure accurately and above pH 9.0 it is not possible to obtain accurate kinetic data; therefore a comparison of the effect of the concentration of KCl and $MgCl_2$ on V_m, ψ_{pHs}, and ψ_{OH-} was made at pH 9.0, and the results are presented in Figure 8. For both KCl and $MgCl_2$ there is a reasonable correlation of V_m with ψ_{OH-}, although the slopes of the two lines are different. For KCl there is also reasonable correlation with ψ_{pHs}, while for $MgCl_2$ there is not a linear correlation of V_m with ψ_{pHs}. It is concluded that ψ_{OH-} more nearly reflects the environment experienced by the enzyme, and that KCl and $MgCl_2$ affect the surface potential by different mechanisms.

Ions in the bulk solution can affect the surface potential in two ways (Davies and Rideal, 1963): either through attraction of oppositely charged ions into the electrical double layer of

TABLE IV: Surface Potential of Dioctanoyllecithin Aggregates.[a]

pH	Water	0.15 M KCl	0.01 M $MgCl_2$	2×10^{-4} M $CaCl_2$
		ψ_{OH^-} (mV)		
9.0	−52.6	−66.3	−38.6	−41.1
9.5	−63.0	−73.2	−43.0	−48.2
10.0	−69.1	−88.8	−47.4	−41.0
10.5	−98.7	−114.8	−69.3	−76.7
		ψ_{pHs} (mV)		
9.0	−53.7	−41.1	−63.0	−60.6
9.5	−68.5	−54.8	−79.5	−78.1
10.0	−78.9	−65.8	−91.8	−87.7
10.5	−94.6	−82.2	−105.5	−101.4
		$\Delta V = (\psi_{OH^-}) - (\psi_{pHs})$		
9.0	+1.1	−25.2	+24.5	+19.5
9.5	+5.5	−18.4	+36.5	+29.9
10.0	+9.8	−41.4	+44.4	+36.7
10.5	−4.1	−32.6	+36.2	+24.7

[a] ψ_{OH^-} is calculated from the rate of OH^- catalyzed hydrolysis (see eq 2 in the text). ψ_{pHs} is calculated from surface pH measurements (see eq 4 in the text).

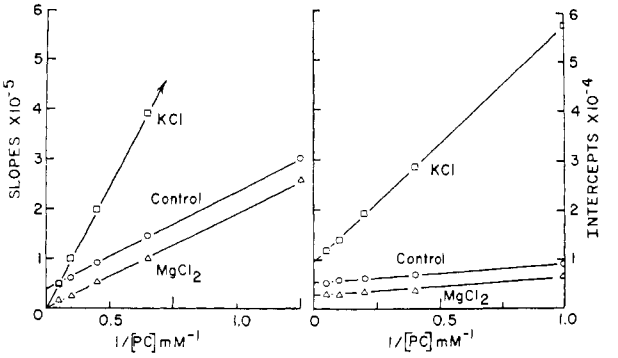

FIGURE 5: Effect of dioctanoyllecithin on the slopes and intercepts of double reciprocal plots of initial velocity as a function of Ca^{2+} concentration at pH 8.0: control, no further additions; $MgCl_2$, addition of 0.01 M $MgCl_2$; KCl, addition of 0.15 M KCl.

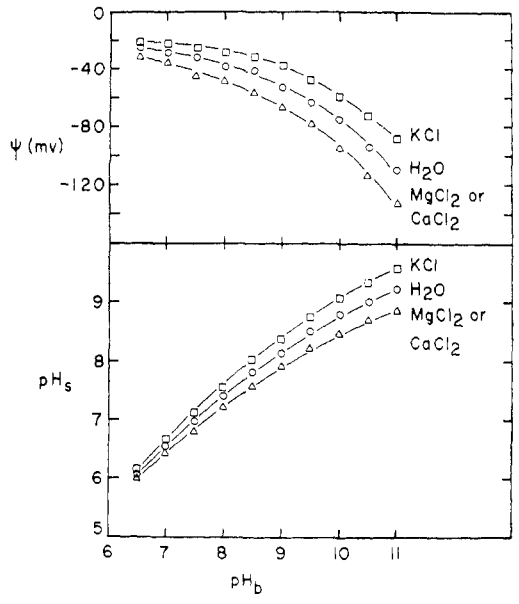

FIGURE 6: Lower panel, surface pH (pH_s) of dioctanoyllecithin aggregates as a function of bulk pH (pH_b) at 45° in H_2O, 0.15 M KCl, 0.01 M $MgCl_2$, or 2×10^{-4} M $CaCl_2$; upper panel, surface potential (ψ) as a function of bulk pH (pH_b).

the particle or by specific absorption of ions into the surface. In the first case, for a spherical particle (Loeb *et al.*, 1961)

$$\psi_{(r)} = \psi_{(a)}(a/r)e^{-\kappa(a-r)} \qquad (9)$$

where $\psi_{(r)}$ = potential at some distance r from a sphere of radius a which has a surface potential $\psi_{(a)}$; κ is the Debye–Huckel distance and is proportional to the square root of the ionic strength (μ). If changes in V_m are due solely to alterations in the electrical double layer, *i.e*, $\psi_{(r)}$, then a plot of ln V_m *vs.* $\sqrt{\mu}$ should be linear.

For ions specifically adsorbed to a charged interface the Stern equation (Davies and Rideal, 1963) can be applied

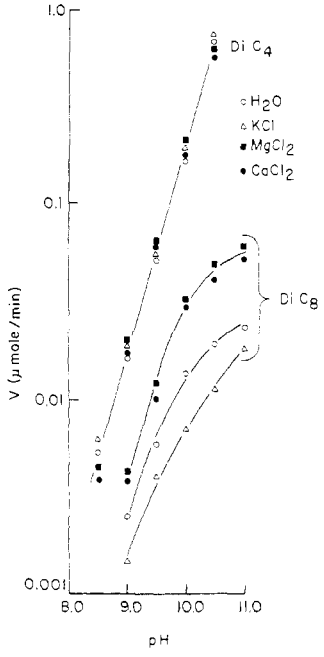

FIGURE 7: Effect of pH on the rate of hydroxide ion catalyzed hydrolysis of dibutyryllecithin (di C_4) or dioctanoyllecithin (di C_8) at 45° in water (○); 0.15 M KCl (△); 0.01 M $MgCl_2$ (■); 2×10^{-4} M $CaCl_2$ (●).

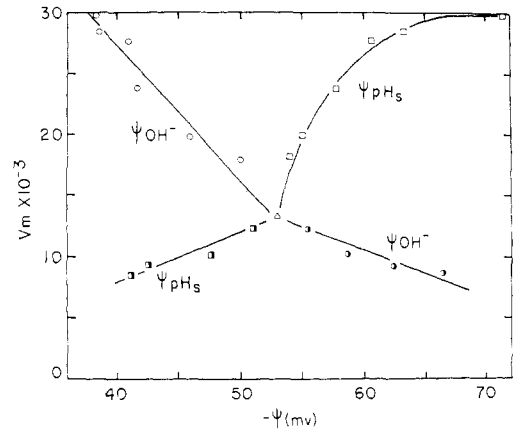

FIGURE 8: Correlation of the effect of the concentration of $MgCl_2$ and KCl on the maximal velocity (V_m) of the phospholipase A_2 catalyzed hydrolysis of dioctanoyllecithin at pH 9.0 with the effect on the surface potential of the dioctanoyllecithin aggregate at pH 9.0: (○) effect of $MgCl_2$ on V_m and the surface potential calculated from the OH^- catalyzed hydrolysis of dioctanoyllecithin; (◑) effect of KCl on V_m and the surface potential calculated from the OH^- catalyzed hydrolysis of dioctanoyllecithin; (□) effect of $MgCl_2$ on V_m and the surface potential calculated from pH_s; (◨) effect of KCl on V_m and the surface potential calculated from pH_s.

(eq 10), where n_c = number of counterions adsorbed, n_s =

$$n_c = (n_s M_c/1000)e^{-Z_2 e\psi/kT} \qquad (10)$$

total number of sites, M = molecular weight of the solvent, and c = bulk concentration of counterions of valency Z_2. This equation is formally an adsorption isotherm and, if for some reason V_m is proportional to n_c/n_s, a plot of $1/V_m$ *vs.* $1/c$ should be linear.

Figure 9 shows a plot of ln V_m *vs.* $\sqrt{\mu}$ at pH 8.0 for solutions containing $CaCl_2$, $CaCl_2$, and KCl, or $CaCl_2$ and $MgCl_2$. In the case of KCl there is a linear correlation of ln V_m and $\sqrt{\mu}$ suggesting a double layer effect. In the case of $CaCl_2$ and $MgCl_2$ the plot is not linear. In Figure 10 the same data are plotted as $1/V_m$ *vs.* 1/[added ion]. In this case both the $CaCl_2$ and $MgCl_2$ data are linear suggesting specific adsorption of these ions, whereas the KCl data are not linear. The conclusion is that KCl affects V_m through an influence on the electrical double layer, whereas the effect of $CaCl_2$ and $MgCl_2$ are through specific adsorption to the interface.

It was shown previously (Wells, 1972) that Ca^{2+} does not bind to dibutyryllecithin; however, the data presented in Table V show that at low concentrations of Ca^{2+} there is

TABLE V: Surface Excess of Calcium Bound to Dioctanoyllecithin Aggregates.[a]

Total Calcium (M)	$_s$(Ca–M)/$_b$(Ca–M)	
	H_2O	0.15 M KCl
1.6×10^{-5}	2.12	1.00
3.2×10^{-5}	1.64	0.93
6.4×10^{-5}	1.32	1.05
12.8×10^{-5}	1.20	0.93
25.6×10^{-5}	1.08	0.96
51.2×10^{-5}	1.04	0.98

[a] $_s$(Ca–M) = ΔA_{600} of the murexide–calcium complex in the presence of dioctanoyllecithin. $_b$(Ca–M) = ΔA_{600} of the murexide–calcium complex in the absence of dioctanoyllecithin.

TABLE VI: Activation Energies for the Hydrolysis of Dioctanoyllecithin and Dibuturyllecithin at pH 8.0 and 45°.[a]

	$\Delta H^{\ddagger}$ (kcal/mol)		$\Delta F^{\ddagger}$ (kcal/mol)		$\Delta S^{\ddagger}$ (eu/mol)	
	V_m	V_m/K_{pc}	V_m	V_m/K_{pc}	V_m	V_m/K_{pc}
Dibutyryllecithin	10.5	9.6	17.7	15.4	−22.6	−18.2
Dioctanoyllecithin						
H_2O	9.6	8.1	12.4	8.6	−8.8	−1.6
0.15 M KCl	9.7	8.5	12.8	9.9	−9.7	−4.4
0.01 M $MgCl_2$	9.5	8.5	12.0	8.5	−7.9	−1.6

[a] Activation energies were calculated either from the temperature dependence of V_m or V_m/K_{pc}.

apparently Ca^{2+} bound to dioctanoyllecithin. This surface excess of Ca^{2+} is eliminated by the presence of KCl. The binding of Ca^{2+} to dioctanoyllecithin is consistent with a negative surface charge. Although Mg^{2+} forms only a weak complex with murexide, the relatively high concentration, compared to Ca^{2+}, precluded making measurements of Ca^{2+} binding in the presence of Mg^{2+}. In agreement with earlier studies (Wells, 1972) there was no effect of dibutyryllecithin on Ca^{2+} binding to murexide.

If the surface charge effects were due to undetected trace contamination by fatty acid or phosphatidic acid one would expect a constant surface charge above pH 6.0. This is not the case. In addition titration of the lecithin from pH 3 to 10 indicated less than 0.01 mol % of material capable of releasing a proton.

Activation Energies. One approach to investigate possible mechanisms which might account for interfacial activation of phospholipase A_2 was to compare activation energies for the hydrolysis of dibutyryl- and dioctanoyllecithins. The results are shown in Table VI. Whether the temperature dependence of V_m or V_m/K_{pc} is determined, the most striking difference between the monomeric and aggregated substrate was found in the entropy of activation. Thus the higher rate of hydrolysis of the aggregated substrate can be attributed to a more favorable entropy of activation. As can be seen in Table VI, this marked decrease in the entropy of activation is seen either in the presence of KCl or Mg^{2+} or with just Ca^{2+} present. It is probable that these data cannot be used to understand the modifying effects of KCl and $MgCl_2$. The linearity of the Arrhenius plots was taken to indicate that no phase changes were occurring in the substrate over the temperature range studied.

Fluorescence Studies. Studies have demonstrated that the binding of dyes, such as ANS, to some proteins lead to enhanced fluorescence of the dye (Edelman and McClure, 1968). Such fluorescence enhancement has been interpreted to indicate binding of the dye to hydrophobic regions on the protein. At pH 8.0 either in the presence or absence of $CaCl_2$ (1×10^{-3} M) no fluorescence enhancement of ANS by phospholipase A_2 was observed.

Discussion

One possible mechanism for interfacial activation of phospholipase A_2 would be adsorption of the enzyme to the interface (a cage effect). The enzyme would be held at the interface and interact with substrate molecules as they diffuse laterally in the interface. Measurements of this lateral diffusion (Lee *et al.*, 1973) set an upper limit to V_m of 1×10^6 mol sec^{-1} mole of enzyme^{-1}. The measured V_m with dioctanoyllecithin

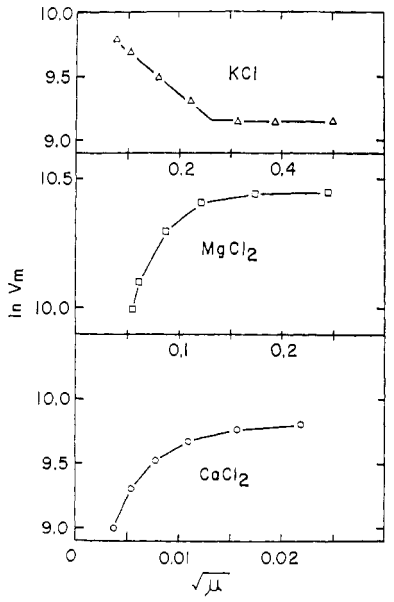

FIGURE 9: Correlation of ln of the maximal velocity of the phospholipase A_2 catalyzed hydrolysis of dioctanoyllecithin at pH 9.0 with the $\sqrt{\mu}$ of added $CaCl_2$, $MgCl_2$, or KCl. For the $CaCl_2$ V_m represents velocity at infinite lecithin at the indicated $CaCl_2$ levels. For KCl and $MgCl_2$ V_m represents velocity at infinite lecithin and $CaCl_2$.

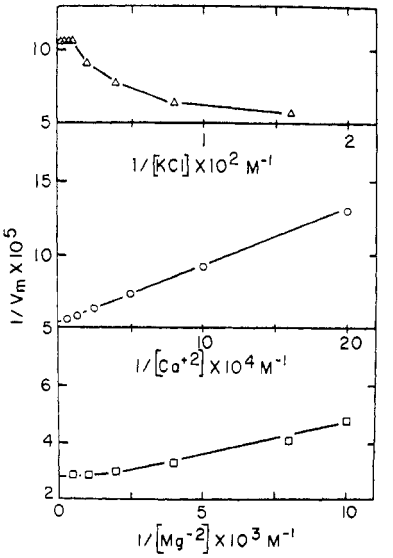

FIGURE 10: Double reciprocal plots of the maximal velocity of the phospholipase A_2 catalyzed hydrolysis of dioctanoyllecithin at pH 9.0 and the concentration of added $MgCl_2$, $CaCl_2$, or KCl. For the $CaCl_2$ V_m represents velocity at infinite lecithin at the indicated $CaCl_2$ levels. For KCl and $MgCl_2$ V_m represents velocity at infinite lecithin and $CaCl_2$.

is well below this value (Table III) and therefore one must seriously consider such a model.

The fact that the inhibition of dioctanoyllecithin hydrolysis by dibutyryllecithin obeys eq 6 argues for a single encounter of the enzyme with the interface for each hydrolytic event. The results suggest that either (1) the enzyme moves freely in and out of the interfacial region since contact with the monomer is not inhibited by the presence of the interface, or (2) at the very least that the enzyme is accessible to monomers in the bulk phase while the enzyme is held in the interface. It seems a moot point then to ascribe the activating effect of substrate aggregation to interfacial binding if the monomers in the bulk phase are as readily accessible to the enzyme as substrate molecules in the interface.

It has been suggested by Verger *et al.* (1973) that pancreatic phospholipase A_2 penetrates the interface. This would presumably require a hydrophobic binding site near the surface of the enzyme. The lack of enhancement of ANS fluorescence by the *C. adamanteus* enzyme taken with the absence of a lag time for hydrolysis of monolayers by this enzyme (Verger *et al.*, 1973) would tend to eliminate penetration of the interface as an important factor in the interfacial activation of *C. adamanteus* phospholipase A_2. Pieterson (1973) has proposed a conformational change might take place upon adsorption of the enzyme to the interface. This has been proposed to enhance the hydrolysis of both monomers and aggregates. The data presented in Table I clearly eliminate this possibility. Although a conformational change in the enzyme might occur upon interaction with the interface, no data to support this suggestion are currently available.

The available data suggest strongly that *C. adamanteus* phospholipase A_2 interacts with the aggregate substrate in a manner which is not fundamentally different than the manner in which it interacts with monomers. How then can one explain the very large rate enhancement attendent upon substrate aggregation?

One possibility that should be considered is a change in the rate-limiting step. If one eliminates the anomalous effects of Ca^{2+} in the absence of added ions, both monomers and aggregates follow an ordered addition of Ca^{2+} and substrate, which suggests that the formation of the enzyme–Ca–substrate complex follows the same mechanism with both types of substrate. The kinetic analysis of the hydrolysis of dibutyryllecithin would suggest that the rate-limiting step might be product release. At an interface ordered release of products would not be feasible (Wells, 1972), and a marked increase in the rate of product release could lead to an increased turnover number for the enzyme. At the present time there does not appear to be a way to confirm or eliminate this possible model for interfacial activation or even to estimate the magnitude of such an effect.

A key observation is the large change in the entropy of activation for the hydrolysis of aggregate substrate compared to monomeric substrates. The negative entropy of activation must arise from loss of rotational and translational degrees of freedom upon formation of the enzyme–substrate complex. There are several ways in which aggregation of the substrate could alter the entropy of activation. (1) The size of the aggregate already reduces the rotational and translational energy of the substrate compared to the monomer. (2) If, as is likely (van Deenen and deHaas, 1963), the glycerophosphoryl group is primarily the site of attachment to the active site, then the surface of the aggregate provides many more productive collisions than the surface of a monomer tumbling in solution.

A third possible mode of activation arises from consideration of recent potential energy calculations for various conformations of lecithin (Gupta and Govil, 1972; McAlister *et al.*, 1973; Vanderkooi, 1973). In the monomer the glycerophosphoryl group can have a large number of conformations; however, imposing the necessity for hydrocarbon chain interactions in the aggregate markedly limits the number of these conformations. Apparently a considerable loss of conformational entropy in the glycerophosphoryl group occurs upon aggregation, and if the conformations assumed in the aggregate are the ones preferred by the enzyme, then one large component of the entropy of activation has already been eliminated during formation of the substrate aggregate.[3]

It is clear from these studies that surface charge effects can modify both the apparent kinetic mechanism of the enzyme and its affinity for the substrate. Although the lecithin molecule is formally electrically neutral, it does contain a permanent dipole. There is a growing body of experimental and theoretical data to support the hypothesis that surface oriented dipoles can give rise to significant electrical effects at an interface (Goldman, 1964; Friedenberg, 1967; Hamel and Zimmerman, 1970; Haydon and Hladky, 1972; Haydon and Myers, 1973). The magnitude and sign of the electrical effects depend on the orientation of the dipoles relative to the plane of the surface. The magnitude of the potential should also be modified by electrical double layer or specific ion adsorption effects.

Two methods were used to evaluate the sign and magnitude of the surface potential. The two methods, surface pH measurements and rate of OH^- hydrolysis, gave different results as seen in Table IV. How can these apparently anomalous results be interpreted?

Figure 11 represents a schematic representation of one of the preferred conformation of lecithin resulting from the studies of McAlister *et al.* (1973). There are two important points: (1) the dipole formed between the $P\text{-}O^-$ and the quaternary nitrogen lies in the plane of the surface of an aggregate and (2) the carbonyl group lies some distance from the surface of the aggregate. Such a model can provide a qualitative explanation of the anomalous data. The OH^- ion must penetrate beyond the surface and its access to the carbonyl group is controlled by a potential, ψ_{OH^-}, at some point arbitrarily shown in Figure 11. On the other hand, the pH indicator is supposed to sit on the surface and be influenced by a different potential, ψ_{pHs}, at or slightly removed from the surface.

If the dipoles are oriented parallel to the surface one would predict that $\psi_{OH^-} = \psi_{pHs}$. Such an orientation would be consistent with the data obtained in H_2O (Table IV). The extended orientation shown in Figure 1 precludes closest approach of the hydrocarbon chains of adjacent molecules. In order for such closest approach to occur the quaternary nitrogen must move out of the plane of the aggregate (down in Figure 11). Such a movement would be resisted by the dipole field of the other molecules in the surface. If, however, the strength of the dipole field were reduced, as for example in the presence of KCl, such a motion might occur due to increased van der Waals interaction of the side chains. Such an orientation would reduce ψ_{pHs} but increase ψ_{OH^-}. An extension of the above reasoning to the effects of Ca^{2+} or Mg^{2+} would suggest that the quaternary nitrogen moves inward from the surface (up in Figure 11). Such an orientation

[3] These entropic effects should not be confused with the entropy effects attendant aggregate formation, which are related to removing the hydrocarbon chains from the aqueous environment.

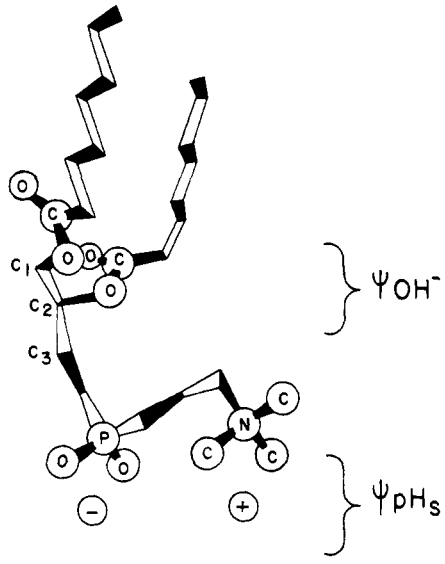

FIGURE 11: Schematic representation of one conformation of dioctanoyllecithin (adapted from McAlister *et al.*, 1973). ψ_{OH^-} is an arbitrary representation of the surface potential measured by the rate of OH^- catalyzed hydrolysis. ψ_{pH_s} is an arbitrary representation of the surface potential measured by surface pH.

would be improbable in an aggregate state. The adsorption of Ca^{2+} or Mg^{2+} to the interface would lead to a net positive charge in the surface near ψ_{OH^-}, but counter Cl^- ion might cause ψ_{pH_s} to become more negative.

These ionic effects can explain the anomalous kinetics observed in the absence of added salts. Since Ca^{2+}, in addition to its catalytic role, also alters the surface potential, the affinity of the enzyme for the substrate changes as the Ca^{2+} increases. Thus kinetic experiments conducted at different Ca^{2+} levels are not comparable. The effect of Mg^{2+} or KCl is to produce a substrate whose properties are not affected by the addition of Ca^{2+}, and the true kinetic mechanism is revealed. The potential effects described above are obviously important in understanding the origin of anomalous kinetic behavior and show that pH studies with aggregate substrates are essentially uninterpretable.

In summary the data presented support a previous suggestion (Wells, 1972), that the interface can markedly affect the apparent kinetic mechanism of phospholipase A_2 and show clearly that kinetic studies with aggregated substrates must be approached with caution (see also Bonsen *et al.*, 1972). It is probable that such effects may be important in understanding the control of enzymes which act intracellularly on lipids held at interfaces (membranes).

Acknowledgments

Mrs. Norma Hewlett provided expert technical assistance. Drs. D. J. Hanahan and J. K. Martin provided helpful comments.

References

Bonsen, P. P. M., deHaas, G. H., Pieterson, W. A., and van Deenen, L. L. M. (1972), *Biochim. Biophys. Acta 270*, 364.

Davies, J. T., and Rideal, E. K. (1963), Interfacial Phenomena, 2nd ed, New York, N. Y., Academic Press.

deHaas, G. H., Bonsen, P. P. M., Pieterson, W. A., and van Deenen, L. L. M. (1971), *Biochim. Biophys. Acta 239*, 252.

Dixon, M., and Webb, E. I. (1964), Enzymes, 2nd ed, New York, N. Y., Academic Press, pp 84–87.

Edelman, G. M., and McClure, W. O. (1968), *Accounts Chem. Res. 3*, 65.

Friedenberg, R. M. (1967), The Electrostatics of Biological Cell Membranes, Amsterdam, North-Holland Publishing Co.

Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, 2nd ed, New York, N. Y., Wiley.

Goldman, D. E. (1964), *Biophys. J. 4*, 167.

Gupta, S. P., and Govil, G. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 27*, 68.

Hamel, B. P., and Zimmerman, I. (1970), *Biophys. J. 10*, 1029.

Haydon, D. A., and Hladky, S. B. (1972), *Quart. Rev. Biophys. 5*, 187.

Haydon, D. A., and Myers, V. B. (1973), *Biochim. Biophys. Acta 307*, 429.

Lee, A. G., Birdsall, N. J. M., and Metcalfe, J. C. (1973), *Biochemistry 12*, 1650.

Loeb, A. L., Overbeek, J. Th. G., and Wiersema, P. H. (1961), The Electrical Double Layer Around a Spherical Particle, Cambridge, Mass., M. I. T. Press.

McAlister, J., Yathindra, N., and Sunderalingam, M. (1973), *Biochemistry 12*, 1189.

Misiorowski, R. L., and Wells, M. A. (1973), *Biochemistry 12*, 967.

Pieterson, W. A. (1973), Ph.D. Thesis, Rijksuniversiteit, Utrecht, The Netherlands.

Roholt, O. A., and Schlamowitz, M. (1961), *Arch. Biochem. Biophys. 94*, 364.

Saito, K., and Hanahan, D. J. (1962), *Biochemistry 1*, 521.

van Deenen, L. L. M., and deHaas, G. H. (1963), *Biochim. Biophys. Acta 70*, 538.

Vanderkooi, G. (1973), *Chem. Phys. Lipids 11*, 148.

Verger, R., Mieras, M. C. E., and deHaas, G. H. (1973), *J. Biol. Chem. 248*, 4023.

Wells, M. A. (1972), *Biochemistry 11*, 1030.

Wells, M. A. (1973), *Biochemistry 12*, 1080.

Wells, M. A., and Hanahan, D. J. (1969), *Biochemistry 8*, 414.

Winsor, P. A. (1968), *Chem. Rev. 68*, 1.