The Activity of Phospholipase A_2 in Reversed Micelles of Phosphatidylcholine in Diethyl Ether: Effect of Water and Cations†

Ronald L. Misiorowski‡ and Michael A. Wells*

ABSTRACT: The apparent specific activity of *Crotalus adamanteus* phospholipase A_2 in reversed micelles of phosphatidylcholine in diethyl ether–methanol (95:5 v/v) is a complex function of substrate, water, and cation concentration. In addition the order in which the reactants are mixed has a profound influence on the activity. If the substrate and water are preequilibrated and the enzyme added in a small volume with $CaCl_2$ the following results are obtained: (1) there is an optimal water concentration at approximately 18 μl/ml; (2) near the water optimum equilibrium order additions of Ca^{2+} and substrate are observed; (3) below the water optimum there is inhibition a high concentrations of Ca^{2+}; (4) the inhibition by Ca^{2+} is competitive with respect to water. If the substrate, water, and $CaCl_2$ are preequilibrated before addition of enzyme, entirely different results are obtained. (1) There is pronounced substrate inhibition, especially at low water and Ca^{2+} concentrations. (2) All reciprocal plots are nonlinear. The solubility of the enzyme in the substrate solution was measured using ^{125}I-labeled protein. Incorporation of the enzyme into the micelle is absolutely dependent on the presence of divalent cations. As opposed to an absolutely specific catalytic requirement for Ca^{2+}, solubilization can be effected by Mg^{2+} and Ba^{2+}. At low water concentrations the enzyme is completely soluble in the micellar solution, and although inactive it is not denatured, since activity can be restored by adding water. At high water concentrations the enzyme cannot be incorporated into the micelle, and is therefore inactive. The activating effect of water is not related to the solubility of the enzyme. In addition to its catalytic role Ca^{2+} was shown to play two important noncatalytic roles: (1) it is necessary for enzyme solubilization; (2) at low water concentrations it forms a complex with the substrate which is enzymatically inactive. On the basis of the data presented it is proposed that at least four distinct micellar species can exist in ethereal solutions of phosphatidylcholine: (1) an anhydrous species, (2) a cation associated species, (3) a moderately hydrated species, and (4) a highly hydrated species. The enzyme is soluble in the first three but not the fourth species; however, only species 3 serves as a substrate. A qualitative explanation of all the kinetic data can be achieved by assuming that the conditions established in the solution before addition of the enzyme affect the distribution of the enzyme among the several species, and that the enzyme can only express its activity when present in micellar species 3.

Considerable evidence exists which suggests that the fine structure of the lipid–water interface exerts an important influence on the activity of phospholipases. This conclusion is supported by studies using three types of interfacial arrangements: monomolecular films (Bangham and Dawson, 1960; Colacicco and Rapport, 1966; Zografi *et al.*, 1972; Verger *et al.*, 1973); bilayers or liposomes (Uthe and Magee, 1971; Jain and Cordes, 1973a); micelles (de Haas *et al.*, 1971; Bonsen *et al.*, 1972; Wells, 1974a). In all of these various systems the primary driving force for the formation of the interface is removal of the hydrocarbon chains from the aqueous environment. To a large extent it is anticipated that the properties of the interfacial region in these systems would be a reflection of the state of the hydrocarbon chains, *e.g.*, whether fluid or solid (Shimshick and McConnell, 1973) or modified by addition of various amphipaths (Bangham and Dawson, 1959; Jain and Cordes, 1973b), etc. Since the polar region of the molecule, which is the site of enzymatic attack, faces into the bulk aqueous solution, it is difficult to study the properties of this region of the interface, although some progress has been made (Wells, 1974a).

An alternative procedure would be to use reversed micelles in organic solvents. In this case the major driving force for aggregation would be removal of the polar head group from the organic solvent into the central core of the micelle. Added water, cations, and enzyme would be expected to concentrate within the central core of the micelle. Under these circumstances the interaction of water and cations with this inward facing interface might be accentuated.

Phospholipase A_2 is known to be extremely active in ethereal solutions of phosphatidylcholine (Hanahan, 1952). Long and Penny (1957) reported an absolute requirement for Ca^{2+} and noted an optimal Ca^{2+} concentration, which depended on the substrate concentration. Long and Penny (1957) and Saito and Hanahan (1962) reported inhibition by high Ca^{2+} concentration; such inhibition has not been observed with monomeric substrates in aqueous solution (Wells, 1972). Wells and Hanahan (1969) reported that the amount of water added to the ether solution critically affected the apparent enzyme activity.

It was therefore of interest to investigate the kinetics of hydrolysis of phosphatidylcholine in ethereal solution, and in particular the effects of substrate, water, and Ca^{2+} concentration. The results of this study in conjunction with the following papers (Poon and Wells, 1974; Wells, 1974b) pro-

† From the Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Arizona 85724. *Received June 6, 1974.* Supported by a grant from the National Science Foundation (GB 35527).

‡ This work is taken from a dissertation submitted by R.L.M. to the University of Arizona in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department of Surgery, College of Medicine, University of Arizona.

TABLE I: Initial Velocities as a Function of Dioctanoylphosphatidylcholine, Water, and Calcium Chloride in Diethyl Ether–Methanol 95:5 (v/v). Method I.[a]

Water (μl/ml)	$CaCl_2$ (mM)	Dioctanoylphosphatidylcholine 2.5	5.0	10.0
		Initial Velocity (μmol per min per mg)		
8.0	0.05	720	2580	3870
	0.10	1350	1980	3420
	0.30	<30	540	2700
12.0	0.05	3150	4860	6390
	0.10	3060	4230	6930
	0.30	2250	3600	4410
15.0	0.05	3870	5580	6840
	0.10	4860	6480	7200
	0.30	6210	6300	7290
18.0	0.05	4590	6570	6930
	0.10	5670	6570	7200
	0.30	6030	6570	8640
23.0	0.05	2430	2970	4770
	0.10	2340	4230	4950
	0.30	1890	3300	5220

[a] Assays were carried out by the addition of water to 2.0 ml of dioctanoylphosphatidylcholine in diethyl ether–methanol 95:5 (v/v) followed by phospholipase A_2 in 2 μl of a calcium chloride solution.

vide new insights into the control of the activity of phospholipase A_2 by the properties of the lipid–water interface.

Materials and Methods

Enzyme purification, substrate (dioctanoylphosphatidylcholine or egg-yolk phosphatidylcholines) preparation, and other general methods have been described (Wells and Hanahan, 1969; Misiorowski and Wells, 1973). Aquasol and ^{125}I were obtained from New England Nuclear (Boston, Mass.). Water used in the enzyme assays was double distilled in a quartz still. Ether was used from freshly opened cans or stored over iron wire. Other chemicals were reagent grade.

Enzyme assays were conducted at room temperature (22–25°) as follows; 2 ml of the substrate in ether–methanol 95:5 (v/v) in a 5-ml volumetric flask was reacted with 0.1 μg of phospholipase A_2 for a specified time. The reaction was terminated by adding 3 ml of absolute ethanol. After addition of 1 drop of 0.1% Cresol Red (aqueous), and a small Teflon-coated stirring bar, the liberated fatty acids were titrated in a N_2 atmosphere with thorough mixing of the solution. An ultraburet (Scientific Industries, Springfield, Mass.) was used to dispense 0.01 N NaOH in 95% ethanol.

The order of addition of water, $CaCl_2$ solution, and enzyme solution to the ethereal solution of substrate markedly influences the apparent activity of the enzyme. One of two standard procedures were followed. Method I: All the water to be used in a specific experiment, except 2 μl, was added to the substrate solution and the contents of the flask was mixed for 15 sec on a Vortex mixer. The enzyme and all the $CaCl_2$ to be present during the reaction were then added in 2 μl; the clear solution was mixed, and set aside for a predetermined time. Method II: All the water to be used in the experiment, except 2 μl, was added to the substrate solution as a $CaCl_2$ solution. After mixing, the enzyme was added in 2 μl of the same $CaCl_2$ solution. Thus the bulk of the $CaCl_2$ was added before the enzyme. In all enzyme assays, specific activity is expressed as μmoles of fatty acid released per min per mg of protein. The protein concentration was determined from the known extinction coefficient at 280 nm (Wells and Hanahan, 1969).

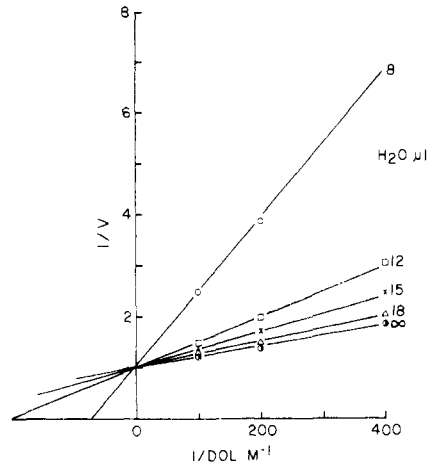

FIGURE 1: Double reciprocal plot of initial velocity as a function of dioctanoylphosphatidylcholine (DOL) concentration at various fixed levels of water and a constant Ca^{2+} concentration of 0.05 mM. These results were obtained by assay method I.

Iodine labeled phospholipase A_2 was prepared by the procedure of Wasserman and Kaplan (1968) using 3.5 μmol of $^{125}I_2$ (specific activity = 760 Ci/mol of I_2) per μmol of enzyme. After dialysis the protein contained 1.6×10^4 cpm of ^{125}I/μg and 0.48 mol of diiodotyrosine/mol as measured by the method of Edelhoch (1962). The specific activity was 0.88 of the native enzyme.

The solubility of ^{125}I-labeled phospholipase A_2 in ether–methanol (95:5, v/v) solutions of phosphatidylcholine was measured as follows. Calcium could not be used in enzyme solubilization studies because the rapid hydrolysis produced lysolecithin at rates which affected the state of the micelle over the time scale of the experiment. It was assumed that either Mg^{2+} or Ba^{2+} would mimic Ca^{2+}. The iodinated protein was diluted with either $MgCl_2$ or $BaCl_2$ and added to 2.0 ml of a phosphatidylcholine solution, which contained the appropriate amounts of water. The solution was mixed vigorously for 25 sec. There was no visible precipitate in any of the solutions. Duplicate 0.3-ml aliquots were transferred to small beakers with a glass syringe. The remaining solution was securely stoppered and centrifuged at 1500*g* for 10 min. Duplicate 0.3-ml aliquots of the supernatant solutions were carefully transferred to small beakers. The solvent was evaporated from all samples in a vacuum oven at room temperature with care to avoid foaming. The samples were counted in duplicate in a Beckman Isomatic Gamma changer attached to a Beckman Model 150 scintillation counter. A full tritium isoset was used as the window to measure the ^{125}I radioactivity. The degree of solubilzation of the enzyme was expressed as the per cent of counts remaining in the supernatant after centrifugation.

Results

General Observations. The rate of hydrolysis was linear with respect to enzyme concentrations up to 0.2 μg/ml. At higher enzyme concentrations, the apparent specific activity

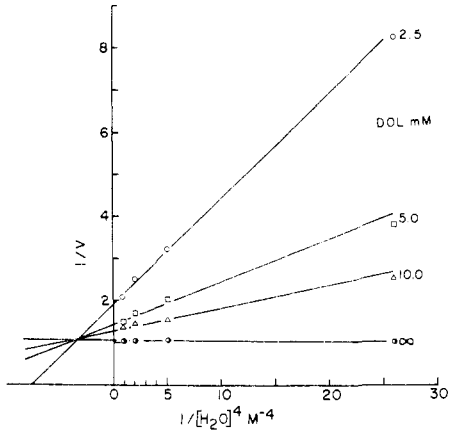

FIGURE 2: Double reciprocal plot of initial velocity as a function of $(H_2O)^4$ at various fixed levels of dioctanoylphosphatidylcholine (DOL) and a constant Ca^{2+} concentration of 0.05 mM. These results were obtained by assay method I.

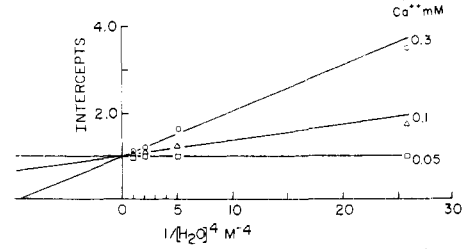

FIGURE 3: The dependence of maximal velocities at infinite phosphatidylcholine concentration (intercepts of double reciprocal plots such as Figure 1) on $(H_2O)^4$ at various fixed levels of Ca^{2+}. These results were obtained by assay method I.

decreased slightly, therefore the enzyme concentration used was 0.1 μg/ml. This concentration yielded a linear response with time up to 15 min. Conditions were chosen such that less than 5% of the substrate was hydrolyzed during the course of the reaction in order to assure that changes in micelle structure due to the liberation of lysophosphatidylcholine and fatty acid had minimal effects on the rates. Specific activities were reproducible within ±10%.

Most studies were conducted with dioctanoylphosphatidylcholine. This phospholipid was found to be an excellent substrate, which was soluble in diethyl ether–methanol under all conditions tested. In some instances egg-yolk phosphatidylcholines were used.

Table I contains the initial rates of hydrolysis at three concentrations of substrate, five water concentrations, and three calcium chloride concentrations using assay method I. Considering only the data obtained at 15 μl of water/ml, double reciprocal plots (Lineweaver and Burk, 1934) of velocity as a function of the substrate concentration at fixed changing concentrations of Ca^{2+} (Cleland, 1963) were linear with all plots intersecting on the ordinate. Double reciprocal plots of velocity as a function of the Ca^{2+} concentration at fixed changing concentrations of substrate were also linear and intersected at a common point to the left of the ordinate. These results, as well as replots of slopes and intercepts, are similar to those reported by Wells (1972) for the hydrolysis of monomeric substrates. By analogy, these data suggest that an equilibrium ordered addition of reactants to the enzyme occurs in reversed micelles of the substrate at 15 μl of water/ml.

V_{max} for the above conditions was 8.3×10^3 μmol per min per mg of enzyme, a value close to that observed for the same substrate in micellar solutions in water (9.8×10^3 at pH 7.0; Wells, 1974a), but considerably faster than the rate of hydrolysis of monomeric substrates (Wells, 1972, 1974a). K_{ica}, the dissociation constant for the enzyme–Ca^{2+} complex, was 0.05 mM, a value close to other estimates (Wells, 1972, 1973, 1974a).

Effect of Water Concentration. Unlike assays conducted in aqueous media, the water concentration in the organic solvent system is limiting and must be considered a reactant. Data on the effect of water concentration on the hydrolytic rates are found in Table I. It is apparent that there is a water optimum at approximately 18 μl of H_2O/ml. If the overall effect of calcium is ignored for the moment, the data at variable phosphatidylcholine and water, at the lowest calcium chloride concentration (0.05 mM), may be treated as a two-substrate reaction. These data are presented in Figure 1 as double reciprocal plots with phosphatidylcholine as the variable substrate and water as the fixed changing substrate. The rate depends on the phosphatidylcholine concentration, as a function of water. Each reciprocal plot, at water concentrations in the region of activation, is linear and intersects at a common point on the ordinate. The inhibiting concentration of water (23 μl/ml) does not conform to this pattern.

When the data are plotted with water as the variable substrate and phosphatidylcholine as the fixed changing substrate, the resulting plots are nonlinear. However, each plot, at constant substrate, approaches linearity when plotted as a fourth-order function of the concentrations of water as shown in Figure 2. The fourth-order dependence of the rate on the water concentration was also noted in methanol–water solutions (Misiorowski and Wells, 1973).

Effect of Ca^{2+} Concentration. The data presented in Table I show that Ca^{2+} causes inhibition at high concentration. The extent of inhibition is greater at low substrate and water concentrations. Double reciprocal plots of velocity *vs.* either substrate concentration or water concentration to the fourth power were linear at all Ca^{2+} concentrations. The effect of Ca^{2+} is seen clearly in Figure 3 where the dependence of the intercepts of double reciprocal plots of velocity and substrate on water concentration at fixed levels of Ca^{2+} are shown. At low Ca^{2+} (0.05 mM) the maximal velocity at infinite substrate concentration is independent of water concentration. However as the Ca^{2+} concentration is increased the maximal velocity at infinite substrate concentration becomes dependent on the water concentration. It should be noted that the maximal velocity at infinite substrate and water concentration is independent of the concentration of Ca^{2+}.

Method II. The observed kinetics are considerably different when calcium ion is added to the reaction mixture by method II. Analysis of these data, Table II, yields primarily nonlinear double reciprocal plots. Figure 4 presents one of these plots as a function of reciprocal velocity and phosphatidylcholine at fixed changing concentrations of Ca^{2+} conducted at 15 μl of water/ml. These data are typical of the general observation of apparent substrate inhibition which occurs at low Ca^{2+}. These data should be compared with those in Table I, at 15 μl/ml of water, which were obtained at essentially the same reactant concentrations by method I. If the data in Figure 4 are replotted as the reciprocal of velocity and the molar ratio of Ca^{2+} to phosphatidylcholine, the resulting plot (Figure 5) is linear except at the highest ratio of phospholipid/Ca^{2+}. Also included in Figure 5 are

TABLE II: Initial Velocities as a Function of Dioctanoylphosphatidylcholine, Water, and Calcium Chloride in Diethyl Ether–Methanol 95:5 (v/v). Method II.[a]

Water (μl/ml)	$CaCl_2$ (mM)	Dioctanoylphosphatidylcholine (mM) 2.5	5.0	10.0
		Initial Velocity (μmol per min per mg)		
8.0	0.004	4,466	1,200	1,400
	0.016	5,000	3,700	1,600
	0.080	5,700	4,200	5,500
	0.160	3,000	3,700	3,800
12.0	0.006	5,933	5,600	3,800
	0.024	9,100	9,800	6,100
	0.120	9,100	10,000	8,200
	0.240	8,400	8,900	7,800
15.0	0.007	7,866	5,600	3,400
	0.030	12,300	10,900	8,400
	0.150	11,400	12,600	12,900
	0.300	10,000	12,400	11,000
18.0	0.009	8,700	7,400	4,800
	0.036	12,200	12,700	12,900
	0.180	11,700	13,900	14,600
	0.360	12,100	12,400	12,400

[a] Assays were carried out by the addition of a calcium chloride solution to 2.0 ml of dioctanoylphosphatidylcholine in diethyl ether–methanol 95:5 (v/v) followed by phospholipase A_2 in 2 μl of the same concentration of $CaCl_2$.

data at 15 μl of water/ml replotted from Table I in a similar format. A comparison of these data indicates that there is an apparent change in kinetic response, which is dependent on the order of calcium addition to the solution of lipid and water.

Enzyme Solubilization by Phosphatidylcholine. In order to assure that the specific activities expressed in the diethyl ether–methanol assays were a function of the total enzyme added, solubilization of phospholipase A_2 by the lipid was conducted as a function of water and calcium concentrations. Since the enzyme is insoluble in diethyl ether–methanol in the absence of lipid, it was expected that the protein which was not solubilized by the lipid could be centrifuged under a low *g* field. Several attempts at conventional determinations of the protein present in the supernatant after centrifugation were unsuccessful due to interference by

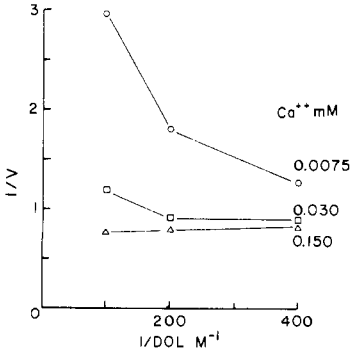

FIGURE 4: Double reciprocal plot of initial velocity as a function of dioctanoylphosphatidylcholine (DOL) concentration at various fixed levels of Ca^{2+} and a constant water concentration of 15 μl/ml. These results were obtained by assay method II.

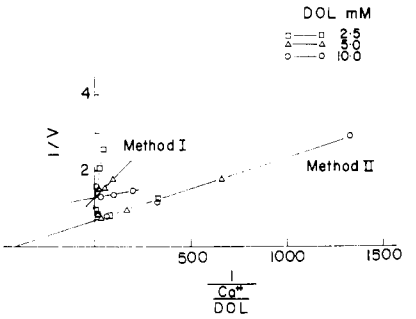

FIGURE 5: Double reciprocal plot of initial velocity as a function of the molar ratio of Ca^{2+} to phosphatidylcholine (DOL). The results obtained using assay method I and II are compared.

lipid. Attempts to extract the enzyme from the organic solvent into an aqueous phase were also unsuccessful. The method which proved to be both reproducible and very sensitive was a radioassay of the enzyme which had been labeled with ^{125}I. Conditions for iodination were chosen such that less than 1 mol of labeled diiodotyrosine/mol of phospholipase A_2 was produced in order to ensure that the enzymatic and solubility properties of the enzyme were minimally altered.

The extent of solubilization of the labeled enzyme as a function of water and Mg^{2+} (substituted for Ca^{2+}) are presented in Table III,a. Essentially all the enzyme is solubilized by egg-yolk phosphatidylcholine at 8 μl of water/ml. This suggests that the low kinetic rates observed at this water concentration are not due to the absence of enzyme in the micelle. The solubilization of the enzyme near the water optimum (18 μl/ml) is almost complete but decreases significantly at 24 μl/ml. The solubilization of the enzyme increases as the cation concentration increases at any water level. This observation does not correlate with the kinetic data in which the specific activities at low water concentrations decrease as the cation concentration is increased; therefore, the inhibition by high calcium at low water is not due to decreased enzyme solubilization.

In the absence of cation, there is virtually no enzyme solubilized into the micelle. These data are found in Table III, b for egg-yolk phosphatidylcholine and Ba^{2+}. The overall solubilization of the enzyme in the presence of Ba^{2+} is essentially identical with the data obtained with Mg^{2+}. There is almost complete solubilization of the enzyme at 8 and 18 μl of water/ml at cation concentrations ranging from 0.05 to 5.0 mM.

An experiment conducted with 5 mM dioctanoylphosphatidylcholine and Ba^{2+} (Table III, c) indicates that there is total enzyme solubilization at cation concentrations ranging from 0.005 to 0.5 mM with water concentrations of 1–18 μl/ml. It should be noted that the enzyme is completely solubilized at 1 μl/ml of water and 5 μM Ba^{2+}. At the highest water concentration, 24 μl/ml, the solubility of the enzyme is reduced drastically. The data presented in Table IV represent a saturation study of enzyme solubilization at 8 μl of water/ml in the presence of 0.1 mM Ba^{2+} and 2.5 mM dioctanoyllecithin. The solubilization is linear from 0.1 to 0.3 μg of phospholipase A_2/ml. The latter concentration represents a threefold higher concentration of enzyme than was used in the kinetic studies.

Since phospholipase A_2 interacts with cations other than Ca^{2+} in diethyl ether–methanol, *i.e.*, Mg^{2+} and Ba^{2+}, as shown in the enzyme solubilization studies, and since both Ca^{2+} and Mg^{2+}, but not Na^+, interact with phosphatidyl-

TABLE III: Solubilization of Phospholipase A_2 by Phosphatidylcholines in Diethyl Ether–Methanol 95:5 (v/v) as a Function of Cation and Water.

Phosphatidylcholine	Cation	Cation Concn (mM)	Water (μl/ml) 1	8	18	24
			% Phospholipase A_2 Solubilized			
(a) Egg yolk (10 mM)	Mg^{2+}	0.05		93	88	38
		0.10		98	93	55
		0.50		102	97	76
(b) Egg yolk (10 mM)	Ba^{2+}	0.0		3	9	11
		0.05		96	93	60
		0.10		108	98	67
		0.05		95[a]	106	86
		5.0		96[a]	96	90
(c) Dioctanoyl- (5 mM)	Ba^{2+}	0.005	105	97	72	9
		0.05	101	101	91	40
		0.10	106	100	98	33[a]
		0.50	99	99	100	1.5[a]

[a] Biphasic system.

cholines, as shown in binding studies conducted in methanol–water (Misiorowski and Wells, 1973), it was of interest to determine the relative specific activity of the enzyme in diethyl ether–methanol as a function of the concentration of Ca^{2+}, Mg^{2+}, and Na^+ at high and low water concentrations. These data are presented in Table V.

If the relative specific activities are based on the reaction at low Ca^{2+} (0.1 mM) and a water concentration near the optimum (16 μl/ml), the relative rate is independent of high Ca^{2+} (0.8 mM), high Mg^{2+} (0.8 mM), or high Na^+ (0.8) concentrations. However, when the same experiment is conducted at low water (6 μl/ml), the specific activity at low Ca^{2+} (0.1 mM) is less than the activity at high water (16 μl/ml). In the presence of high Ca^{2+}, the relative activity is still lower due to inhibition by Ca^{2+} as previously observed (Table I). The relative activity at low Ca^{2+} plus a high concentration of Mg^{2+} (0.8 mM) is the same as the relative rate at high Ca^{2+} alone. Thus the inhibition observed at low water and high cation concentrations is not a specific effect of Ca^{2+}, but is due to a nonspecific effect of divalent cations, since the relative rate observed at low Ca^{2+} plus high monovalent cation (Na^+) is the same as the relative rate observed at low Ca^{2+} alone.

Stability of the Enzyme. One possible explanation for the low activity observed at low water concentrations is that some of the enzyme is denatured and therefore the apparent specific activity is less. As can be seen from the data in Table VI, the enzyme is completely stable in ethereal solutions of phosphatidylcholine at low water content for at least 15 min.

TABLE IV: Solubility of Phospholipase A_2 in Solutions of Dioctanoylphosphatidylcholine in Diethyl Ether–Methanol 95:5 (v/v) containing 0.1 mM $BaCl_2$ and 8 μl of Water/ml as a Function of Phospholipase A_2 Added.

μg of Phospholipase A_2 Added	μg of Phospholipase A_2 Solubilized
0.10	0.10
0.30	0.27
0.70	0.57
1.50	1.35

Discussion

In light of the obvious complexities of this system, perhaps the most remarkable aspect of the data is that linear reciprocal plots are obtained at all, let alone under most of the conditions described in Table I. We feel that these data hold the key to understanding the system, since any model proposed must account for these linear relationships, as well as, explaining the nonlinear data shown in Table II. In an inverted micellar solution, substrate concentration cannot be interpreted in terms of single molecules interacting with the enzyme. In addition the concentration of added water and cations is rather vaguely defined. We have chosen to express concentration in terms of the whole reaction volume, although we understand that water, cations, and enzyme do not distribute uniformly throughout the solution, but are concentrated to varying degrees within the micelles.

TABLE V: Relative Specific Activity of Phospholipase A_2 in Diethyl Ether–Methanol 95:5 (v/v) as a Function of Various Cations at High and Low Water Concentrations.[a]

Cations Added	Water (μl/ml) 6	16
	Rel Specific Activity	
0.1 mM Ca^{2+}	0.55	1.0
0.8 mM Ca^{2+}	0.27	0.94
0.1 mM Ca^{2+} + 0.8 mM Mg^{2+}	0.28	0.95
0.1 mM Ca^{2+} + 0.8 mM Na^{2+}	0.53	1.0

[a] Each assay contained 5 μmol of dioctanoylphosphatidylcholine in diethyl ether–methanol 95:5 (v/v) and 0.1 μg of phospholipase A_2. The cation or cations in combination were added with the enzyme. All relative specific activities were based on the reaction which contained 0.1 mM Ca^{2+} and 16 μl of water/ml.

TABLE VI: The Stability of Phospholipase A_2 in Ethereal Solutions of Phosphatidylcholine at Low Water Concentrations.[a]

Expt	Specific Activity
1. Substrate mixed with 20 μl of H_2O, then add enzyme. Assay after 5 min	6000
2. Substrate mixed with 2 μl of enzyme, assay after 20 min	10
3. Substrate mixed with 2 μl of enzyme	
(a) Stand 5 min, add 20 μl of H_2O, assay after 5 min	5800
(b) Stand 10 min, add 20 μl of H_2O, assay after 5 min	6200
(c) Stand 15 min, add 20 μl of H_2O, assay after 5 min	6000

[a] Two milliliters of a 10 mM solution of egg-yolk phosphatidylcholine in diethyl ether–methanol 95:5 (v/v) was mixed with 20 μl of water and 2 μl of a phospholipase A_2 solution (50 μg/ml in 0.1 M $CaCl_2$) as outlined. The fatty acids produced were determined after the time interval indicated.

At the present time we do not have sufficient information to express concentration in terms of the actual environment in which the reaction occurs.

Since the enzyme is insoluble in ether, we cannot suppose that a classical enzyme–substrate interaction occurs, which would lead to an increase in the concentration of E–S with increasing substrate concentration. Physical studies (Poon and Wells, 1974) show that the size of the micelle does not increase appreciably with increasing lipid concentration, therefore increasing substrate concentration probably does not increase the number of substrate molecules present in the micelles containing the enzyme. In addition, at any substrate concentration, there is a several fold excess of micelles over enzyme molecules. It is possible that increased substrate concentration might lead to an increased rate of replenishment of substrate molecules within the micelle containing the enzyme. However, such a proposal would necessarily assume that the rate of reaction approaches the rate of movement of molecules into and out of the micelles. We consider this an unlikely possibility.

The model we propose to account for the observed kinetic behavior is based on the following considerations. (1) Regardless of the manner in which the experiment is conducted or the concentration of cation, there is a consistent activating effect of increasing the water content of the solution up to a certain point, and then a decrease in enzyme activity at higher water concentrations. We consider it most likely that the effects of water are related to hydration of the substrate since (a) the activity of phospholipase A_2 correlates with the amount of water bound to phosphatidylcholine (Poon and Wells, 1974); (b) the fourth-order dependence on water found here coincides with a similar dependence on water found in methanol–water solutions, where it was shown that phosphatidylcholine binds four molecules of water (Misiorowski and Wells, 1973); (c) the amount of water required to observe activity apparently far exceeds the amount of water that the protein could bind; (d) the protein is completely soluble in the lipid solution at very low water concentrations, and although it is inactive, it is not denatured.

In order to account for the activating and inhibitory effects of water we proposed the existence of two distinct hydration states

$$PC + mH_2O \rightleftharpoons PC(H_2O)_m$$

$$PC(H_2O)_m + n(H_2O) \rightleftharpoons PC(H_2O)_p \quad (p = n + m)$$

The active substrate is $PC(H_2O)_m$, and the activating effect of water relates to formation of this species from the anhydrous species (PC). $PC(H_2O)_p$ is an inactive substrate species and the inhibitory effect of water relates to its formation. This substrate form apparently cannot solubilize the enzyme (Table III; see also Poon and Wells, 1974). Another factor may be related to the reduced solubility of this highly hydrated species (Misiorowski and Wells, 1973).

(2) Although Ca^{2+} is absolutely required for the activity of phospholipase A_2 (Wells, 1972), it is apparent that Ca^{2+} exerts additional influences in the system under discussion. Similar noncatalytic effects of Ca^{2+} were noted when micellar substrates were used in water (Wells, 1974a). We reported previously (Misiorowski and Wells, 1973) that the Ca^{2+} binds to phosphatidylcholine in organic solvents and that this binding is diminished in the presence of water. Further, we showed that the phosphatidylcholine–Ca^{2+} complex was not a substrate for phospholipase A_2, and that this inhibitory effect of Ca^{2+} was mimicked by Mg^{2+}. The data presented in this paper strongly support the suggestion that in addition to its catalytic role Ca^{2+} and other divalent cations play two noncatalytic roles. First, divalent ions are absolutely required for solubility of the enzyme in the micelle. This most likely represents a charge neutralization phenomena similar to that reported by Gitler and Montal (1972). The second effect is inhibition of the enzyme at high cation and low water concentrations. This effect most likely relates to the observation that divalent cation–phosphatidylcholine complexes are enzymatically inactive.

Consider first the results obtained using assay method 1. The species equilibrium established before addition of enzyme depends solely on the water content of the solution and at each water concentration a unique species distribution exists (Poon and Wells, 1974). As the water content increases, the relative concentration of the reactive micelle reaches a maximum and then decreases. The absolute concentration of the reactive micelle will depend on the actual substrate concentration used. Since the enzyme is added in a solution rich in Ca^{2+}, it is proposed that most of the Ca^{2+} is solubilized in the same micelle as the enzyme. Under optimal conditions (15 μl of H_2O/ml) we observe kinetics which are consistent with equilibrium ordered addition of reactants (Wells, 1972). Since the relative concentration of the reactive species is maximal and its absolute concentration increases with increasing substrate concentration, linear reciprocal plots are observed. In addition under these conditions the content of Ca^{2+} within the micelle increases giving the linear reciprocal plots.

At lower water concentrations or higher Ca^{2+} concentrations, the primary factor determining the rate of reaction is the distribution of enzyme between various micelles. Increasing water favors partition of the enzyme into the reactive micelle, up to the point where high water favors formation of the less reactive (inactive) highly hydrated form. Increasing the Ca^{2+} concentration, at constant water, favors formation of the Ca^{2+}-rich micelle, which is also inactive. This analysis predicts that the inhibition by Ca^{2+} should be competitive with respect to water, as is seen in Figure 3.

The observed linear kinetics are not the result of a classical enzyme–substrate type mechanism, but the result of partitioning the enzyme between various micelles with different reactives. Since the partition depends on the concentration of the reactants, the observed response is analogous to classical kinetics.

Method II creates an entirely different situation prior to enzyme addition. Since Ca^{2+} is added before the enzyme, we must consider the effects of both water and Ca^{2+} on the distribution of micellar species, and also the availability of Ca^{2+} to the enzyme, which is added in a solution relatively poor in this cation. At a low water concentration, formation of the Ca^{2+}-rich micelle is favored (Misiorowski and Wells, 1973), and solubility data suggest that the enzyme is readily incorporated into this species (Table III). At higher substrate concentrations the amount of this species would increase. Further, as the substrate concentration increases, the amount of Ca^{2+} available to the enzyme in the reactive species is decreased due to an increase in the concentration of the Ca^{2+}-rich micelle. This analysis predicts substrate inhibition at low water arises from a combination of lowered Ca^{2+} in the reactive micelle and less enzyme in the reactive micelle. The amount of cation that can be solubilized within a micelle is limited (Misiorowski and Wells, 1973); therefore, as the cation concentration increases, more may partition into the reactive micelle. It is reasonable to suggest that the relative distribution of Ca^{2+} would depend on the cation to lipid ratio. These results are obtained by method II. The lessened substrate inhibition seen at higher water concentrations probably reflects a decreased importance of the Ca^{2+}-rich micelle, although the amount of Ca^{2+} available to the enzyme may still be limiting.

While the above analysis has obvious limitations, we feel that it serves to emphasize an important concept, namely that the primary factor affecting the activity of phospholipase A_2 in this system is the physical state of the substrate micelles. In such a situation it is apparent that detailed mechanistic studies are impractical; however, the enzyme may serve as a sensitive indicator of altered physical states within the micelles. Thus a comparison of the activity of various substrates may provide a great deal of information about the differences in the physical properties of those substrates, and little, if any, information, about the inherent specificity of the enzyme. In the succeeding articles (Poon and Wells, 1974; Wells, 1974b) considerable data are provided concerning the physical properties of the phosphatidylcholine micelles and the nature of the water bound to these micelles. We feel that a combination of all of these data represent the most extensive attempt to date to correlate the activity of a lipolytic enzyme with the physical properties of its substrate. We also realize that we have only begun to scratch the surface of this important and intriguing problem.

References

Bangham, A. D., and Dawson, R. M. C. (1959), *Biochem. J. 72,* 486.

Bangham, A. D., and Dawson, R. M. C. (1960), *Biochem. J. 75,* 133.

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

Cleland, W. W. (1963), *Biochim Biophys. Acta 67,* 104.

Colacicco, G., and Rapport, M. M. (1966), *J. Lipid Res. 7,* 258.

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

Edelhoch, H. (1962), *J. Biol. Chem. 237,* 2788.

Gitler, C., and Montal, M. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 28,* 329.

Hanahan, D. J. (1952), *J. Biol. Chem. 195,* 199.

Jain, M. K., and Cordes, E. H. (1973a), *J. Membrane Biol. 14,* 101.

Jain, M. K., and Cordes, E. H. (1973b), *J. Membrane Biol. 14,* 119.

Lineweaver, H., and Burk, P. (1934), *J. Amer. Chem. Soc. 56,* 658.

Long, C., and Penny, I. F. (1957), *Biochem. J. 65,* 382.

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

Poon, P. H., and Wells, M. A. (1974), *Biochemistry 13,* 4928.

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

Shimshick, E. J., and McConnell, H. M. (1973), *Biochemistry 12,* 2351.

Uthe, J. F., and Magee, W. L. (1971), *Can. J. Biochem. 49,* 776.

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

Wasserman, P. M., and Kaplan, N. O. (1968), *J. Biol. Chem. 243,* 720.

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

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

Wells, M. A. (1974a), *Biochemistry 13,* 2248.

Wells, M. A. (1974b), *Biochemistry 13,* 4937.

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

Zografi, G. Verger, R., and de Haas, G. H. (1972), *Chem. Phys. Lipids 7,* 185.