Competition between Cations and Water for Binding to Phosphatidylcholines in Organic Solvents†

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ABSTRACT: Experiments are reported which show that phosphatidylcholines can bind Ca^{2+}, Mg^{2+}, and Ce^{3+} in anhydrous methanol. In all three cases there is 1 mol of cation bound per mol of phosphatidylcholine with the following association constants: Ca^{2+} = 0.0286 mM^{-1}, Mg^{2+} = 0.0244 mM^{-1}, Ce^{3+} = 2.5 mM^{-1}. Glycerophosphorylcholine also binds 1 mol of Ce^{3+} with an association constant of 2.5 mM^{-1}. Cation binding to phosphatidylcholines in methanol is competitively inhibited by water. The binding of four water molecules per phosphatidylcholine prevents the binding of the cations. Evidence is also presented which suggests that ion binding and the inhibition of binding by water are not related to the aggregation state of the lipid. Kinetic studies with *Crotalus adamanteus* phospholipase A_2 in methanol–water solutions show that the hydrated form of phosphatidylcholine is the substrate, whereas the anhydrous form and the calcium complex are not substrates. In diethyl ether–methanol solutions phosphatidylcholine can solubilize both calcium and water. Excess calcium or water can precipitate the lipid from diethyl ether–methanol solutions. There is a synergistic effect of water and calcium in precipitating the lipid. In chloroform solutions neither water nor calcium precipitates the lipid. In benzene solutions water precipitates the lipid, but calcium acts antagonistically to water. Nuclear magnetic reasonance studies failed to reveal any differences between the structure of the polar head group of the lipid in the anhydrous form or the hydrated form, which could account for the interaction with calcium. The potential importance of the weak, environmentally sensitive, interaction between calcium and phosphatidylcholine is discussed in light of recent theories on the role of calcium in excitable membranes.

The potential role of phospholipids in ion transport and membrane permeability has stimulated considerable research on the interactions between phospholipids and cations (for recent reviews, see Triggle, 1972, and Vandenheuvel, 1971). Although acidic phospholipids interact strongly with a variety of cations, several studies have shown that phosphatidylcholine does not interact with cations in aqueous solution (Hauser and Dawson, 1967; Barton, 1968; Dawson and Hauser, 1970; Wells, 1972). Several studies have appeared on the interaction of phosphatidylcholines with water in a variety of solvents (Demchenko, 1961; Elworthy and McIntosh, 1964a,b; Hendrikson, 1970; Walter and Hayes, 1971; Haque *et al.*, 1972) as well as with films of phosphatidylcholine (Elworthy, 1961, 1962; Salsbury *et al.*, 1972). Generally there is an increase in the size of the micelle upon water binding in organic solvents and some data suggest that there are at least two distinguishable hydration shells. To date no data have appeared on the effects of cations on the hydration of phosphatidylcholines in organic solvents.

During an analysis of the *Crotalus adamanteus* phospholipase A_2 catalyzed hydrolysis of phosphatidylcholines in organic solvents, we observed a strong inhibition by calcium ions under certain conditions. Such inhibition has also been noted by Long and Penny (1957) and by Saito and Hanahan (1962). Long and Penny (1957) also noted that the concentration of calcium which gave maximal activity depended on the concentration of the substrate. We have confirmed this latter observation and also noted that the inhibitory effect of calcium depends on the concentration of water in the organic solvent. Such inhibition by calcium was not observed in a study of the hydrolysis of dibutyryllecithin in aqueous solutions (Wells, 1972).

We therefore postulated that under certain conditions calcium could bind to phosphatidylcholines and that this binding was diminished in the presence of water. This paper presents the results of studies carried out to test this hypothesis, and defines some of the conditions under which such interactions can take place.

Materials

C. adamanteus phospholipase A_2, *sn*-glycero-3-phosphorylcholine, and hen's egg yolk phosphatidylcholines were prepared as described (Wells, 1972). 1,2-Dioctanoyl-*sn*-glycero-3-phosphorylcholine[1] was prepared by the method of Cubero Robles and van den Berg (1969) and purified as previously outlined (Saunders and Wells, 1969; Wells 1972). The final product analyzed for 5.92% phosphorus (calcd 5.87) and had an $[\alpha]_{546}^{25}$ +10.2° (*c* 4.5, $CHCl_3$–CH_3OH,1 :1). Cerium chloride was from Ventron Corp (Beverly, Mass.), LH-20 Sephadex from Pharamacia (Picataway, N. J.), ^{45}Ca and ^3H_2O from New England Nuclear (Boston, Mass.), and deuterated methanol (D_4, 99.5% D) and deuterated diethyl ether (D_{10}, 99.5% D) from Mallinckrodt Chemical Works (St. Louis, Mo.). EM Gel OR-750 was from Waters Associates (Framingham Mass.) and Aquasol from New England Nuclear (Boston, Mass.). Other materials were reagent grade and used without purification.

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[1] According to the tentative rules for lipid nomenclature (IUPAC) these compounds should be named as derivatives of *sn*-glycero-3-phosphorylcholine, but for simplicity they will be called lecithins.

Methods

Analytical methods have been described (Wells, 1972). Fluorescence measurements were made with a Perkin-Elmer fluorescence spectrophotometer Model MPF-2A. Enzyme assays were carried out in a total volume of 1.0 ml using 13.5 μg of phospholipase A_2 and 25 mM dioctanoyllecithin. The reaction was terminated at the end of 10 min by the addition of 3 ml of ethanol, and the liberated fatty acids were titrated immediately in an atmosphere of N_2 with 0.01 N NaOH in 10% H_2O in ethanol. The base was dispensed with an ultraburet (Scientific Industries, Springfield, Mass.), and cresol red was used to detect the end point. The measurement of activity was reproducible to within 5–10%.

Ion binding studies were carried out by gel filtration on Sephadex LH-20 using a 0.9 × 26 cm LC-9MA column and a sample injection valve with a 0.25-ml sample loop (Chromatronic Inc., Berkeley, Calif.). A flow rate of 0.05 ml/min was maintained with a Marriott flask. After the column was equilibrated with the desired solution containing various amounts of $CaCl_2$, 0.1 μCi of ^{45}Ca/100 ml, and other additions as appropriate, 0.25 ml of a 100 mM lecithin solution in the equilibrating solvent was placed on the column. Approximately 0.2-ml fractions were collected in tared flasks and analyzed for phosphorus and radioactivity. Sufficient counts were accumulated so that the expected counting error was ±0.5%. The average specific activity of all non-phosphorus-containing fractions (generally 25 fractions) was determined along with the standard deviation. Those phosphorus-containing fractions in which the radioactivity was at least three times greater than the standard deviation determined above ($P < 0.01$) were used to calculate the calcium bound to the lecithin.

Water binding to phosphatidylcholine in diethyl ether–methanol solutions, containing ^3H_2O, was measured by gel filtration on EM gel OR-750. The same column described above was used. Since solvent evaporation was a potentially serious problem, the sample was injected onto the column through a sample injection valve (see above). This permitted the entire operation to be conducted in a closed system, except for collection of the sample. In order to collect the samples without solvent evaporation, the eluate was collected directly in 5.0 ml of Aquasol. The amount of eluate collected in each vial was determined by weight. Radioactivity and phosphorus were measured.

The solubilities of lecithin and $CaCl_2$ (at 22–23°) were measured in chloroform, benzene, or diethyl ether. To 0.95 ml of the lipid solution was added 50 μl of a methanolic solution of $CaCl_2$ containing 0.05 μCi of ^{45}Ca, and when appropriate, water. The final composition of the mixture was 95% solvent, 5% methanol, with varying amounts of lecithin, $CaCl_2$, and water. After mixing, the samples were let stand for 10 min with occasional mixing, then the samples were centrifuged for 20 min at 1500g. Aliquots of the supernatant solution were analyzed for phosphorus and radioactivity.

The amount of water that can be solubilized by lecithin in diethyl ether and the transfer of Ca^{2+} from water to ether in the presence of lecithin were determined. Various amounts of hen's egg yolk lecithin were dissolved in 1.0 ml of diethyl ether. In one experiment 1.0 ml of water containing ^3H_2O (25 μCi/mg of water) was added, and in a second the same water containing 0.01 M $CaCl_2$ was used. In the third experiment unlabeled water was used and the solution contained 0.01 M $CaCl_2$ and 0.05 μCi of ^{45}Ca/μmol of Ca^{2+}. The tubes were sealed and the two-phase system was mixed on a Vortex mixer for 20 min. The tubes were then placed in a 25.0 ± 0.02° water bath and left for 24 hr to allow phase separation. The ether phase was analyzed for phosphorus and radioactivity, and the aqueous phase was analyzed for phosphorus.

Nuclear magnetic resonance spectra were obtained in a Varian HA-100 spectrometer at a probe temperature of 35°, using Me_4Si (in a capillary tube) as an internal standard.

Analysis of Binding Data. If calcium binds to phosphatidylcholine (PC),[2] then in general terms the equilibrium can be represented as follows[3]

$$PC + nCa \rightleftharpoons PC\text{–}Ca_n \tag{1}$$

where n represents the number of calcium molecules bound per mole of lecithin. n can have any value, *e.g.*, 0.5, 1, 2, etc. The association constant for the interaction can be written as

$$k_{Ca} = \frac{[PC\text{–}Ca_n]}{[PC][Ca]^n} \tag{2}$$

If we define $\bar{v}$ as the moles of Ca bound per mole of PC, *i.e.*

$$\bar{v} = \frac{n[PC\text{–}Ca_n]}{[PC] + [PC\text{–}Ca_n]} \tag{3}$$

then combination of eq 2 and 3 leads to

$$\bar{v} = \frac{nk_{Ca}[Ca]^n}{1 + k_{Ca}[Ca]^n} \tag{4}$$

$$\frac{1}{\bar{v}} = \frac{1}{nk_{Ca}[Ca]^n} + \frac{1}{n} \tag{5}$$

$\bar{v}$ is determined from the excess Ca found in the lecithin containing fractions from gel filtration experiments. [Ca] refers to the concentration of calcium used to equilibrate the column. A plot of $1/\bar{v}$ *vs.* $1/[Ca]^n$ should be a straight line. k_{Ca} can be determined from the intercept with the $1/[Ca]^n$ axis. n is determined by plotting the binding data as various functions of $1/[Ca]^n$ until a value of n is found which gives a straight-line plot.

Once k_{Ca} and n are known the binding of other molecules can be investigated by determining their effect on $\bar{v}$ for calcium binding. Assuming that $n = 1$ for calcium binding, and water competes with calcium for binding to PC, there would be two simultaneous equilibria

$$PC + Ca \rightleftharpoons PC\text{–}Ca \tag{6}$$

$$PC + mH_2O \rightleftharpoons PC\text{–}H_2O_m \tag{7}$$

and the association constants

$$k_{Ca} = \frac{[PC\text{–}Ca]}{[PC][Ca]} \tag{8}$$

$$k_{H_2O} = \frac{[PC\text{–}H_2O_m]}{[PC][H_2O]^m} \tag{9}$$

[2] Abbreviations used are: PC, phosphatidylcholine or lecithin; GPC, glycerophosphorylcholine.

[3] This derivation should not be confused with small molecule–macromolecule interactions (Thompson and Klotz, 1971).

TABLE I: Binding of Calcium to Phosphatidylcholine in Methanolic Solutions.[a]

Experimental Conditions	$\bar{\nu}$ [b]
I. Dioctanoyllecithin in anhydrous methanol, and	
6.25 mM Ca^{2+}	0.145 ± 0.012[c]
12.5 mM Ca^{2+}	0.258 ± 0.004
25.0 mM Ca^{2+}	0.375 ± 0.010
50.0 mM Ca^{2+}	0.580 ± 0.015
100.0 mM Ca^{2+}	0.700 ± 0.012
II. Hen's egg yolk lecithin in anhydrous methanol, and	
4.0 mM Ca^{2+}	0.113 ± 0.016
12.0 mM Ca^{2+}	0.233 ± 0.080
III. Dioctanoyllecithin, 12.5 mM Ca^{2+}, and	
5.0% water[d]	0.240 ± 0.012
10.0% water	0.120 ± 0.016
20.0% water	0.015 ± 0.005
IV. Dioctanoyllecithin and 12.5 mM Ca^{2+} in anhydrous methanol, and	
25.0 mM NaBr	0.260 ± 0.017
12.5 mM $MgCl_2$	0.206 ± 0.009

[a] Determined by gel filtration on Sephadex LH-20 as detailed in the text. [b] Average moles of Calcium bound per mole of phosphatidylcholine. [c] Standard deviation within a run. [d] Per cent by volume.

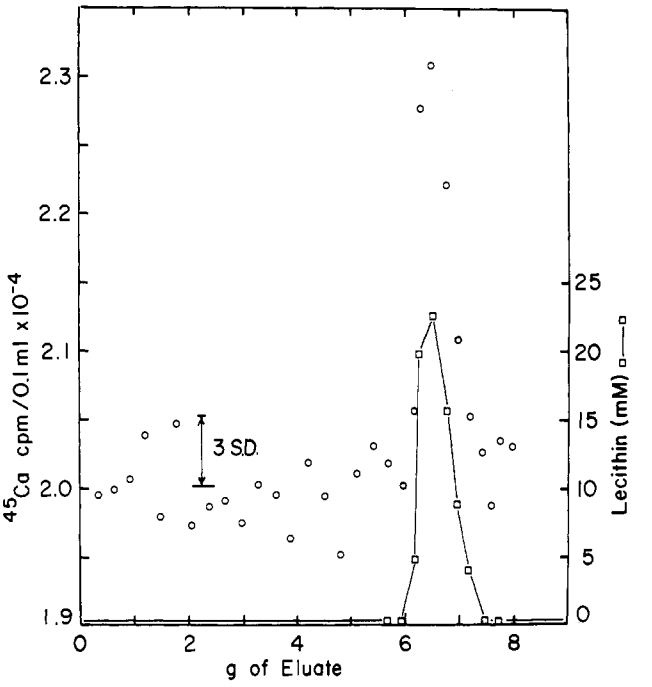


FIGURE 1: Binding of calcium to dioctanoyllecithin in methanol. Gel filtration of dioctanoyllecithin on LH-20 equilibrated with 50 mM $CaCl_2$ in anhydrous methanol. The open circles represent the radioactivity of ^{45}Ca present in each fraction. The squares represent the amount of lecithin present in each fraction. "3 S.D." refers to three times the standard deviation of the average ^{45}Ca radioactivity in all non phosphorus-containing fractions.

where [PC], [Ca], and [H_2O] refer to the concentration of the unbound species. If we define $\bar{\nu}$ as in eq 3 then

$$\bar{\nu} = \frac{[\text{PC–Ca}_n]}{[\text{PC}] + [\text{PC–Ca}] + [\text{PC–H}_2\text{O}_m]} \quad (10)$$

$$\bar{\nu} = \frac{k_{Ca}[Ca]}{1 + k_{Ca}[Ca] + k_{H_2O}[H_2O]^m} \quad (11)$$

which can be rearranged to

$$\log \left\{ k_{Ca}[Ca]\left(\frac{1}{\bar{\nu}} - 1\right) - 1 \right\} = \log k_{H_2O} + m \log [H_2O] \quad (12)$$

A plot of the left side of eq 12 as a function of log water should be a straight line with slope m. This formulation also applies to any situation in which there is competition between calcium and any material for binding to phosphatidylcholine.

Results

One calcium binding experiment using gel filtration on Sephadex LH-20 is shown in Figure 1. The average value of $\bar{\nu}$ was 0.580. The results of experiments carried out in anhydrous methanol are plotted in Figure 2. The data are a linear function of $1/Ca^n$ if $n = 1$. Included in Figure 2 are data obtained both with dioctanoyl and egg lecithin. There is not a significant difference between the results obtained with these two lecithins. The value of k_{Ca} is 0.0286 mM^{-1}.

When calcium binding experiments were carried out in the presence of water, there was a decrease in $\bar{\nu}$, as shown in Table I. These data are plotted in Figure 3 according to eq 12. The various lines in Figure 3 are drawn assuming that one to six water molecules are being bound. The data are best fitted assuming that four water molecules are involved. The calculated association constant for water, k_{H_2O}, is 0.0017 M^{-4}.

The results presented in Table I show that Mg^{2+} can also

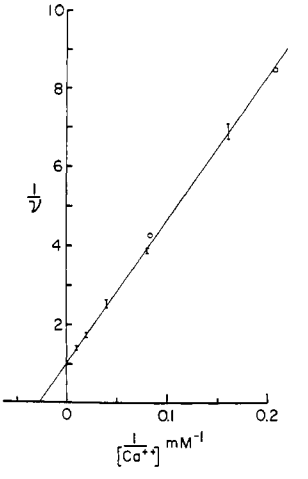


FIGURE 2: Determination of n and k_{Ca} for calcium binding to lecithin in anhydrous methanol. The data for calcium binding have been plotted as a function of $1/[Ca]^n$ with $n = 1$. The open circles refer to experiments carried out with hen's egg holk lecithins, and the bars to those carried out with dioctanoyllecithin. The line is a least-squares line through the dioctanoyllecithin data.

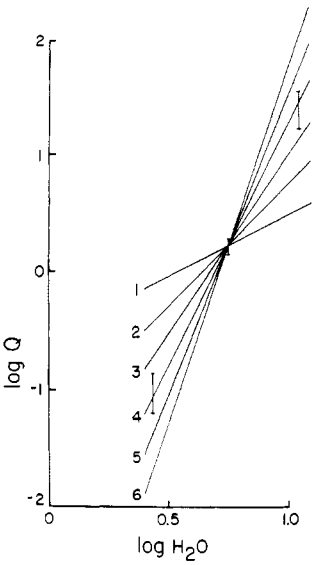


FIGURE 3: Determination of the number of water molecules bound to lecithin in methanol. The inhibition of calcium binding to dioctanoyllecithin by water is plotted according to eq 12 (see text). Log Q refers to the left side of eq 12. The bars represent the range of calculated values using the experimental data. The lines labeled 1–6 are drawn assuming 1–6 water molecules are bound.

bind phosphatidylcholine in anhydrous methanol with an association constant, k_{Mg}, of 0.0244 mM^{-1}. No evidence for binding of sodium ions was obtained.

In all the experiments described above the elution volume of the lecithin was 5.25 ± 0.25 ml. This is presumptive evidence that the state of aggregation of the lipid is not altered in the various experiments.

Recently it has been suggested that rare earth cations might be useful probes of calcium binding sites in proteins (Birnbaum *et al.*, 1970). Cerium is a highly fluorescent member of this series, and when bound to ligands, the fluorescence of cerium is quenched (Jørgensen, 1962). Several experiments were conducted which show that cerium can bind to lecithins and GPC in anhydrous methanol. Figure 4 shows the decrease in cerium's fluorescence caused by GPC. Similar results were obtained using dioctanoyllecithin and egg lecithins. Other ions, such as F^- and CrO_4^{2-}, which are strong ligands of Ce^{3+} (Jørgensen, 1962), also caused quenching of fluorescence in methanolic solutions. On the other hand I^-, which is an extremely weak ligand, did not affect the fluorescence. In water, F^- and CrO_4^{2-} still caused quenching of fluorescence, but GPC had no effect, even at concentrations 100-fold higher than those which caused quenching in methanol. Using fluorescence to estimate the concentration of free cerium, it was determined that GPC, dioctanoyllecithin, and egg lecithin each bind 1 mol of cerium with an association constant of approximately 2.5 mM^{-1}. The binding of cerium by GPC in methanol was diminished as the water concentration was increased.

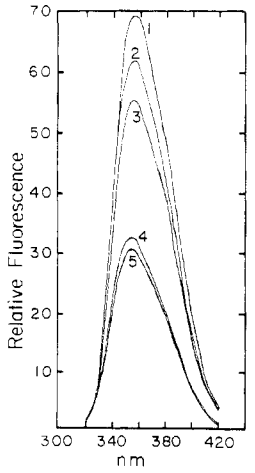


FIGURE 4: Quenching of the fluorescence of cerium by glycerophosphorylcholine. Fluorescence emission spectra were recorded while activating at 317 nm. All solutions contain 2.0 × 10^{-4} M $CeCl_3$ in methanol. Curve 1: no added GPC; curve 2: plus 0.5 × 10^{-4} M GPC; curve 3: plus 1.1 × 10^{-4} M GPC; curve 4: plus 2.9 × 10^{-4} M GPC; curve 5: plus 8.5 × 10^{-4} M GPC. All solutions were in a final volume of 3.0 ml.

TABLE II: Effect of Water on the Solubility of Dioctanoyllecithin and $CaCl_2$ in Diethyl Ether–Methanol.[a]

Experiment	Lecithin in the Supernatant Soln (μmol/ml)	$CaCl_2$ in the Supernatant Soln (μmol/ml)
I. Lecithin, no Ca^{2+}		
μl of water/ml		
0	9.8	
5	9.5	
10	9.9	
15	9.9	
II. $CaCl_2$, no lecithin		
μl of water/ml		
0		0.310
5		0.073
10		0.027
15		0.022
III. Lecithin and $CaCl_2$		
μl of water/ml		
0	9.7	5.03
5	9.8	5.06
10	9.9	4.91
15	0.1	0.20

[a] Each mixture contained 10 mM dioctanoyllecithin or 5 mM $CaCl_2$ (with 0.01 μCi of ^{45}Ca) or both lecithin and $CaCl_2$. The solvent composition was diethyl ether–methanol (95:5, v/v). After addition of the indicated amount of water, the solution was mixed, allowed to stand 10 min, and then centrifuged for 20 min at 1500g. Aliquots of the supernatant solution were removed for phosphorus analysis and radioactivity measurement.

Further evidence for the interaction of water and calcium for binding to lecithin is found in Table II. It can be seen that $CaCl_2$ has a very limited solubility in the solvent alone, but in the presence of lecithin all the added calcium was solubilized. As the water concentration is increased from 0 to 15 μl per ml, there is no effect on the solubility of the lecithin, but the solubility of $CaCl_2$ is decreased. The ability of the lecithin to solubilize $CaCl_2$ is not affected by water concentrations up to 15 μl/ml. At this point both the lecithin and $CaCl_2$ become insoluble.

TABLE III: Solubility of Dioctanoyllecithin in Various Solvents as a Function of the Concentration of Water and $CaCl_2$.[a]

	Water Added (μl/ml)						
	0	5	10	15	20	25	30
Solvent	Lecithin in Supernatant Solution (μmol/ml)						
I. Diethyl ether[b]							
$CaCl_2$ (mM)							
0.0	3.93	4.03	4.08	3.91	3.91	3.98	1.47[c]
1.0	3.91	3.95	3.92	4.04	2.80[c]		
2.0	3.93	3.90	2.05[c]	0.83[c]			
3.0	3.96	1.96[c]	0.95[c]				
4.0	1.35[c]						
5.0	0.34[c]						
II. Benzene[b]							
$CaCl_2$ (mM)							
0.0	3.94	1.30[c]					
5.0	3.90	2.90[c]					
III. Chloroform[b]							
$CaCl_2$ (mM)							
0.0	3.95	3.89[c]	4.14[c]				
5.0	3.90	4.02[c]	3.91[c]				

[a] To 0.95 ml of a 4 mM solution of dioctanoyllecithin in the indicated solvent was added 50 μl of a methanolic solution of $CaCl_2$ to give the desired final concentration of $CaCl_2$. Where indicated water was added, and the solution was mixed and allowed to stand for 10 min. After centrifugation at 1500*g* for 20 min, aliquots of the supernatant solution were taken for phosphorus analysis. [b] $CaCl_2$ was soluble in the benzene and chloroform solvent systems in the absence of added lipid, but not in the ether solvent system. [c] Biphasic system.

It can been seen in Table III that in the absence of $CaCl_2$, between 25 and 30 μl of water causes precipitation of the lecithin. As the $CaCl_2$ concentration is increased, the amount of water required to precipitate the lecithin decreases. Examination of the precipitate by thin layer chromatography showed no hydrolysis or other detectable alteration in the lecithin. It was observed that the precipitate was completely soluble in methanol, as would be expected from the data on calcium binding in methanol.

$CaCl_2$ was completely soluble in benzene and chloroform (Table III), and there was no precipitation of lecithin even in the presence of excess $CaCl_2$ under anhydrous conditions. In the chloroform solutions, it was not possible to demonstrate precipitation of lecithin by water even in the presence of $CaCl_2$. In benzene there was precipitation of the lecithin by water alone, but in contrast to the ether system, there was less precipitation when both $CaCl_2$ and water were present.

Figure 5 presents the solubilization of water and Ca^{2+} in ethereal solutions of egg lecithin, utilizing transfer experiments from either pure water or 0.01 M $CaCl_2$ in water. The data show that lecithin in ether can solubilize 60 mol of water/mol, but less than 0.01 mol of Ca^{2+}/mol. These data again demonstrate that water inhibits the interaction of lecithin and calcium in certain circumstances. No lecithin was detected in the aqueous phase in these experiments.

Kinetic Studies. In order to investigate the possible significance of the various forms of lecithin in methanol–H_2O–$CaCl_2$ mixtures, kinetic studies were carried out using *C. adamanteus* phospholipase A_2. Figure 6 presents these results. Two points should be emphasized. First the rates are higher in 20% H_2O in methanol than in 10% H_2O in methanol; second, there is inhibition at high calcium concentrations, but the inhibition is more pronounced in 10% H_2O in methanol than in 20% H_2O in methanol.

In aqueous solutions Mg^{2+} has no effect on phospholipase A_2; however, as shown in Table IV, Mg inhibits the reaction in 10% water in methanol. Under the same conditions, NaBr had no effect. When the water concentration was increased to 20% there was little effect of Mg^{2+} except at the highest concentration tested (50 mM).

The activating effect of water was further investigated using low concentrations of $CaCl_2$, as shown in Figure 7. Reciprocal velocities have been plotted *vs.* the reciprocal of water to the first power and the fourth power. A straight line is obtained

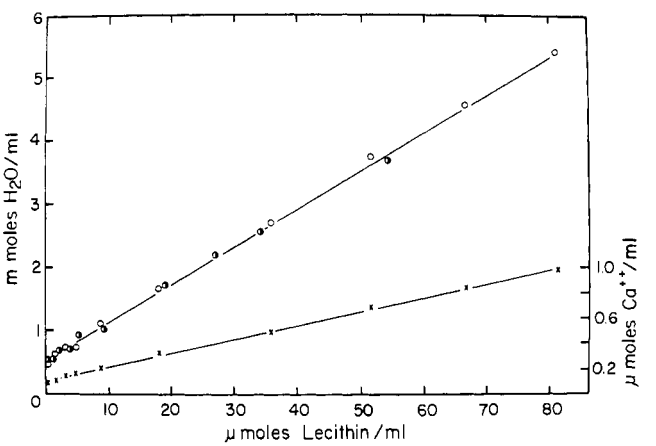


FIGURE 5: Solubilization of water and calcium by hen's egg yolk lecithins in diethyl ether. Ethereal solutions of lecithin were equilibrated against water or aqueous 0.01 M $CaCl_2$. The open circles represent the water solubilized when water was used, and the half-filled circles to the water solubilized when 0.01 M $CaCl_2$ was used. The X's represent the calcium solubilized when 0.01 M $CaCl_2$ was used.

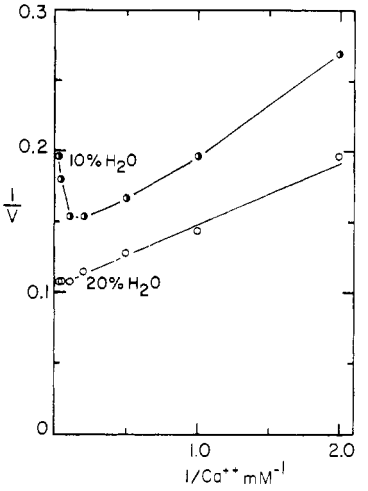


FIGURE 6: Reciprocal plots of specific activity of phospholipase A_2 as a function of the $CaCl_2$ concentration at two fixed levels of H_2O in methanol. Assays were carried out using 25 mM dioctanoyllecithin and 13.5 μg of *C. adamanteus* phospholipase A_2.

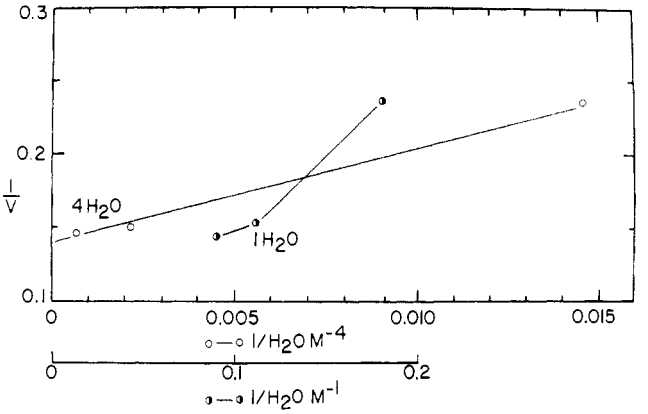


FIGURE 7: Reciprocal plots of specific activity of phospholipase A_2 as a function of the water concentration in methanol. Assays were carried out using 25 mM dioctanoyllecithin, 1 mM $CaCl_2$, and 13.5 μg of *C. adamanteus* phospholipase A_2. The open circles assume four water molecules are required in the reaction, while the half-filled circles assume one water molecule is required.

only when it is assumed that the activation by water requires four water molecules. This is consistent with the data presented in Figure 3.

Assuming that there are three species of lecithin present in methanol–H_2O–$CaCl_2$ mixtures, namely an anhydrous species, a hydrated species which binds four water molecules, and a species which binds one calcium, and using the association constants reported in Table I, one can calculate the concentration of each species present as a function of water and $CaCl_2$. The results are shown in Figure 8. In 20% water the concentration of the hydrated species is high and changes little as the $CaCl_2$ concentration increases. The concentration of the species which binds calcium is low. In 10% water there is a marked decrease in the concentration of the hydrated species as the calcium concentration increases, and a concomitant increase in the concentration of the species binding calcium.

The lower rates found in 10% water could be accounted for by the lower concentration of the hydrated species, if it were the substrate for the reaction, and/or the increased concentration of the species binding calcium, if it were an inhibitor. In order to gain some information on this point, the ratio of the concentration of the hydrated species present in 20% water to that in 10% water at different calcium concentrations was calculated from the data in Figure 8. The ratio of specific activities was also calculated for the same conditions. These two ratios are plotted in Figure 9. There is a positive correlation between an increased concentration of the hydrated species and an increased specific activity. The correlation coefficient of the line in Figure 9 is 0.97. A similar treatment of data using the concentration of the species binding calcium is also present in Figure 9. There is a decrease in rate as the concentration of the species binding calcium increases; however, the largest change in concentration occurs at a calcium concentration where the relative rate is little changed. At high concentrations of calcium one could not rule out the possible inhibitory effect of the calcium-bound species, although the inhibition could equally well be explained by a simple lowering in concentration of the hydrated species.

H_2O Binding in Diethyl Ether. One experiment which demonstrates water binding to egg lecithin is presented in Figure 10. The value of $\bar{v}$ was 0.80. Due to the high concentration of water required to saturate the lecithin micelle, we were not able to carry out a complete study. However, this experiment does demonstrate that water binds to the lecithin micelle in diethyl ether.

Nuclear Magnetic Resonance Studies. If Ca^{2+} binding caused a change in the conformation of the phosphorylcholine residue, we anticipated a change from the gauche conformation (Sundaralingam, 1968; Dufourcq and Lussan, 1972). However, nuclear magnetic resonance studies on phosphatidylcholines in methanol-d_4 or diethyl-d_{10} ether under a variety of conditions failed to provide any data which could be used to interpret possible conformational changes of the polar head group.

TABLE IV: Effect of Mg^{2+} and Na^+ on the Activity of Phospholipase A_2 under Various Conditions.[a]

Conditions	Sp Act. (μmol/min per mg)
I. Methanol–water (90:10, v/v)	
Control (no additional cations)	5.1
Addition of 1 mM $MgCl_2$	5.1
Addition of 10 mM $MgCl_2$	4.6
Addition of 25 mM $MgCl_2$	4.2
Addition of 50 mM $MgCl_2$	3.7
Addition of 25 mM NaBr	5.1
Addition of 50 mM NaBr	5.1
II. Methanol–water (80:20, v/v)	
Control (no additional cations)	7.0
Addition of 10 mM $MgCl_2$	7.0
Addition of 25 mM $MgCl_2$	7.0
Addition of 50 mM $MgCl_2$	6.5

[a] Assays were carried out using 25 mM dioctanoyllecithin, 1 mM $CaCl_2$, and 13.5 μg of phospholipase A_2 in a 1-ml reaction volume. The cations were added as methanol solutions. The reaction time was 10 min and the liberated fatty acids were titrated with 0.01 N NaOH.

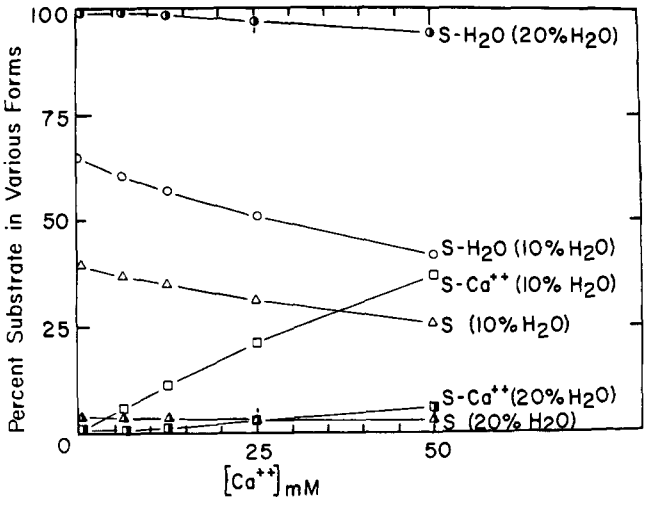


FIGURE 8: Per cent of lecithin present in solution as the anhydrous species (S), hydrated species (S–H_2O), and calcium complex (S–Ca^{2+}) as a function of the calcium concentration at two levels of water (10 and 20%). The data were calculated assuming $k_{Ca} = 0.0286$ mM^{-1}, $k_{H_2O} = 0.0017$ M^{-4}, and a solution containing 25 mM dioctanoyllecithin.

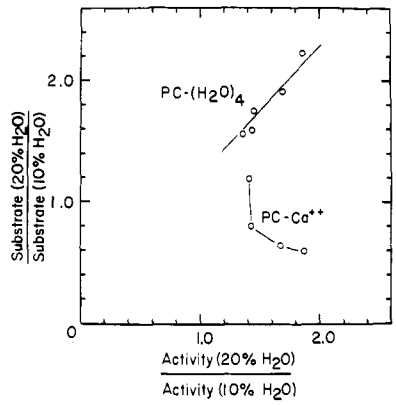


FIGURE 9. Correlation between phospholipase A_2 activity and the relative concentration of the hydrated substrate species [PC–$(H_2O)_4$] or the calcium complex (PC–Ca^{2+}). At a given calcium concentration the ratio of enzymatic activity in 20% H_2O in methanol to that in 10% water in methanol was calculated from the data in Figure 6. For the same calcium concentration the ratio of the substrate species present as PC–$(H_2O)_4$ or PC–Ca^{2+} in 20% H_2O in methanol to that in 10% water in methanol was calculated from the data in Figure 8. These two ratios are plotted in this figure. The least squares line through the PC–$(H_2O)_4$ data has a correlation coefficient of 0.97.

Discussion

The data presented are consistent with the hypothesis that lecithins can bind cations, such as calcium and magnesium under certain conditions, and that there is competition between cations and water for binding. The most convincing data are those obtained by gel filtration on Sephadex LH-20 (Figures 1, 2, and 3; Table I). Before attempting a molecular interpretation of the data, there are several factors which must be taken into consideration. (1) Is the interaction between the lecithin and calcium monomolecular, *i.e.*, do individual molecules of lecithin interact with calcium or is the calcium somehow solubilized inside a lecithin aggregate? (2) Does water interact with the lecithin directly in the sense outlined above, or does water affect some alteration in the aggregation state such that calcium can no longer be solubilized? (3) Is the effect of water on the lecithin or upon the calcium?

Recent data (Kellaway and Saunders, 1970a,b) show that hen's egg yolk lecithins are monomeric in methanol, ethanol, and propanol, although earlier studies (Elworthy and McIntosh, 1961) indicated that egg yolk lecithins were trimers in methanol, and that dipalmitoyllecithin was a monomer in methanol (Elworthy and McIntosh, 1964a,b). Kellaway and Saunders (1970b) found little tendency toward aggregation until the water concentration approached 50% in propanol, whereas Elworthy and McIntosh (1964a,b) found considerable aggregation in 16% water in methanol. Whether the differences in these results can be ascribed to the differences in methods of molecular weight estimation is not known at the present time. Since the elution volumes of both dioctanoyl and egg yolk lecithins were the same in methanol, and since the elution volume was not altered by varying the composition of the solvent, we assume that the lecithins are predominantly monomeric in all the gel filtration experiments. Certainly there cannot be a large change in the aggregation state.

Two additional observations strongly suggest that the binding of calcium cannot be ascribed solely to the presence or absence of aggregates. In aqueous solutions there is no binding of calcium to dispersed sols (Barton, 1968), monomolecular films (Hauser and Dawson, 1967) or to monomeric solutions of dibutyryllecithin (Wells, 1972). The fact that glycerophosphorylcholine binds cerium in methanol, but not in water, indicates that the presence of the fatty acid chain is not required either for the binding of the cation or for the inhibition of cation binding by water. Cerium also binds to lecithins as well as to glycerophosphorylcholine in methanol. We conclude that in the methanol–water system the binding of cations and the inhibition of cation binding by water are properties of the phosphorylcholine moiety, and not a result of different aggregation states with altered abilities to solubilize calcium.

Schneider and Strehlow (1962) and Daniel and Maatman (1963) have studied the selective solvation of calcium in methanol–water solutions. There is some disagreement between these two groups as to whether there is selective solvation by water or not. Schneider and Strehlow (1962) suggest that

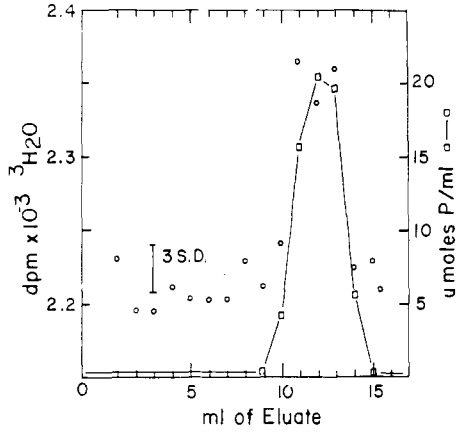


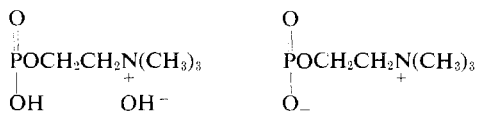
FIGURE 10: Binding of water to lecithin in diethyl ether. Gel filtration of hen's egg yolk lecithins on a column of EM Gel-750 equilibrated with ether–methanol (95:5, v/v) and containing 4 μl of H_2O/ml. The open circles represent the radioactivity of ^3H_2O in each fraction. The squares represent the amount of lecithin in each fraction. "3 S.D." refers to three times the standard deviation of the average ^3H_2O radioactivity in all non-phosphorus-containing fractions.

there are 15 molecules in the solvation shell of calcium and that the number of water molecules would be 0 in pure methanol, 2.0–2.5 in 5% water in methanol, 4.0–4.5 in 10% water in methanol, and 10–11 in 20% water in methanol. Daniel and Maatman (1963) suggest that the solvation shell reflects the composition of the solvent. Using either set of data, there is no direct correlation between the increased number of water molecules in the hydration shell of the calcium and the decreased binding of calcium to lecithins. Therefore, we tend to eliminate selective solvation of calcium by water as an important contributing factor to the effect of water on the binding of calcium to lecithins.

The number of water molecules which are required to inhibit calcium binding, four, is reasonably consistent with other studies on water binding to lecithins. Elworthy (1961, 1962) studying absorption of water vapor by lecithin films found three hydration layers. The two most tightly bound layers constitute four to six molecules of water. Salsbury *et al.* (1972), using deuteron magnetic resonance studies of lecithin–water systems, estimate that the lamellar phase binds four water molecules tightly and the gel phase 9. Hendrikson (1970) estimated that twelve water molecules could be bound in CCl_4 solutions. Walter and Hayes (1971) detected two hydration shells in benzene solutions, the first consisting of two to three molecules of water and the second of four to six molecules of water.

In their analysis of the crystal structure of glycerophosphorylcholine, Abrahamsson and Pascher (1966) noted that the quaternary nitrogen is approximately tetrahedrally coordinated to four oxygen atoms. Although there is no water in these crystals, it is possible to construct models of the polar head group in which this tetrahedral arrangement is formed by four water molecules. One of many possible arrangements has two of the water molecules hydrogen bonded to the unesterified oxygens of the phosphate group. In addition one water oxygen lies very close to the quaternary nitrogen. Whether this or any other hypothetical structure bears any resemblance to fact is impossible to determine. Also it is not possible to determine whether such structures have sufficient energy to prevent binding of Ca^{2+}.

The polar head group of lecithins can be formulated in two ways. Form A might predominate in media of low dielectric



constant since the charges are separated by a smaller distance than in form B. The consequence of such a structure would be that calcium would be exchanging for a H^+ on the phosphate group. There has been evidence presented to the effect that phosphatidylcholines contain one very tightly bound water (Baer, 1953; Abramson *et al.*, 1965). We have been unable to detect any alterations of the polar head groups by nuclear magnetic resonance (nmr) under the various conditions described in this paper, which would rule out gross conformational changes on Ca^{2+} or water binding.

The interaction between cations and water can also be seen in the composition of the two cadmium chloride complexes of glycerophosphorylcholine. If the complex is formed at low water, it has the composition $(GPC)_2(CdCl_2)_3$, whereas after recrystallization, from aqueous ethanol, the composition is $GPC{-}CdCl_2{-}3H_2O$ (Baer and Kates, 1948). The crystal structure of this latter compound (Sundaralingam and Jensen, 1965) shows that the conformation of the polar head group is the same as in the free diester (Abrahamsson and Pascher, 1966).

The free energy of formation of the Ca^{2+}–PC complex in anhydrous methanol can be estimated to −0.9 kcal/mol. This is some three to four times smaller than the formation of Ca^{2+}–acidic phospholipid complexes in water. No estimates have been made of the formation of Ca^{2+}–acidic phospholipid complexes in organic solvents, but they could reasonably be expected to be considerably higher.

Feinstein (1964) has reported on the ability of phosphatidylcholines to transport calcium from methanol–water into chloroform; however, we find no transfer of calcium from water into ether. The amount of water which can be solubilized by lecithin in ether is considerably greater than has been reported in aromatic solvents (Demchenko, 1961; Elworthy and McIntosh, 1964a,b).

The interaction of lecithins and water, lecithins and calcium, and lecithins and both water and calcium depends on the solvent in which the interaction is studied. Thus $CaCl_2$ is virtually insoluble in diethyl ether in the absence of added lecithin, whereas in chloroform and benzene it is soluble without added lipid. In diethyl ether and benzene, when the capacity of the system to solubilize water is exceeded, the lipid comes out of solution, whereas in chloroform it does not. In diethyl ether the presence of $CaCl_2$ reduces the solubility of the lipid in the presence of water, in benzene it increases the solubility, and in chloroform it has no effect. There is obviously a complex interplay between the nature of the solvent, the amount of water, calcium, and lipid. The effect of these various factors on the aggregation state of the lipid is currently under investigation.

A further indication of significant structural differences between the three forms of lecithin presumed to exist in methanolic solutions, *i.e.*, anhydrous form, hydrated form, and calcium complex, can be found in the response of phospholipase A_2. There is a clear dependence on the concentration of water. This dependence is fourth order and correlates well with the formation of the hydrated species. We therefore propose that the enzyme is able to form a productive complex only with the hydrated species. Whether the enzyme can bind to the anhydrous species or the calcium complex is still unclear. The data presented suggest that the inhibition by calcium correlates with the decreased concentration of the hydrated active form of the substrate. Wells (1972) has presented evidence for an ordered addition of calcium and the lecithin to the enzyme. It would appear unlikely that a lecithin molecule which is already complexed to calcium could add to the enzyme–calcium complex. The nature of the structural alteration involved in going from the anhydrous species to the hydrated species is unknown. If the form which exists in the absence of water has structure A (see earlier), then the ability to interact with the enzyme might be reduced if the oxygen on the phosphate must be ionized for the interaction to take place (van Deenan and DeHaas, 1963). The ability of calcium to interact with this species may also be related to the state of the ionization of the phosphate oxygen.

In recent years several workers have attempted to formulate models of the excitable membrane, which involve both the polar head group of phospholipid and calcium (Goldman, 1964; Wang, 1970; Hamel and Zimmerman, 1970). While a detailed consideration of these models is beyond the scope of this paper (see Triggle, 1972, for a recent review), we would like to point out the potential importance of the observations

reported in this paper. All of the models have in common a conformational alteration in the membrane which leads to a decreased interaction of the phospholipid with calcium and allows entry of sodium into the nerve. The interaction between calcium and phosphatidylcholine would seem ideally suited to serve these models. The combination of the weak, environmentally sensitive interactions between calcium and phosphatidylcholine and the stronger interactions between calcium and acidic phospholipids, as recently discussed by Ohki and Papahadjopoulos (1970), may allow a more complete description of the excitable membrane.

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