Binding of Calcium to Phosphatidylcholines as Determined by Proton Magnetic Resonance and Infrared Spectroscopy†

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ABSTRACT: The interactions of calcium, magnesium, and the rare earth cations, cerium, neodymium, and praseodymium, with phosphatidylcholines were studied by proton magnetic resonance and infrared spectroscopy. The calcium-induced chemical shifts for the various protons of phosphatidylcholine were C_α choline > C_β choline > $N(CH_3)_3$ > C_3 glycerol. No significant chemical shifts were observed for the C_1 and C_2 glycerol protons. None of the acyl chain protons were affected by the presence of calcium. Analysis of the salt-induced chemical shifts yielded binding curves with an excellent fit with the theoretical. The vicinal coupling constants for the various protons of phosphatidylcholine did not appear to change in the presence of calcium. The lanthanide-induced isotropic shifts for the protons of phosphatidylcholines followed the order C_β choline > C_3 glycerol > C_α choline > $N(CH_3)_3$. Examination of the P=O stretching band (1150–1300 cm^{-1}) of phosphatidylcholines by differential infrared spectroscopy showed that this band shifted to shorter wavelengths in the presence of calcium. The site of calcium binding to phosphatidylcholines as deduced from the proton magnetic resonance and infrared data is discussed in light of the high specificity for calcium in enhancing the amine-catalyzed methanolysis of phosphatidylcholines.

Misiorowski and Wells (1973) reported that phosphatidylcholine could bind calcium, magnesium, and cerium ions in methanolic solutions. Wells (1974) subsequently reported that calcium enhanced the amine-catalyzed methanolysis of phosphatidylcholines. In spite of a similar binding constant for complexation to phosphatidylcholine, magnesium was very ineffective in promoting the amine-catalyzed methanolysis reaction, and cerium, which binds more tightly, was completely without effect in this reaction. Analysis of the kinetics of this methanolysis reaction suggested that the reactive intermediate was a calcium–phosphatidylcholine–amine complex. It was also suggested that an enzyme bound calcium–phosphatidylcholine complex should be considered as a possible intermediate in the mechanism of action of phospholipase A_2.

As a part of a study to determine the mode of binding of phosphatidylcholine to phospholipase A_2, it was of interest to assess the site of calcium binding to phosphatidylcholine in the model system. In addition, a comparison of the interactions of calcium, magnesium, and rare ions might allow us to understand the specificity of the metal ion enhancement of the methanolysis reaction.

In this paper, we present the results of proton magnetic resonance and infrared spectroscopic studies of the interaction of calcium, magnesium, and rare earth ions with phosphatidylcholine methanolic solutions.

Experimental Section

Materials. The preparations of hen's egg yolk phosphatidylcholines (Wells and Hanahan 1969), *sn*-glycero-3-phosphorylcholine (Wells, 1972),1,2-diacetyl-*sn*-glycero-3-phosphorylcholine, and 1,2-dibutyryl-*sn*-glycero-3-phosphorylcholine (Misiorowski and Wells, 1973) have been previously described. Calcium chloride (anhydrous) was from Allied Chemical (Morristown, N.J.). Cerium chloride, neodymium chloride, praseodymium chloride, and anhydrous magnesium chloride were from Ventron Corp. (Beverly, Mass.). All salts were dried at 200° for 48 hr and stored over phosphorus pentoxide at 0.1 mm. Deuterated methanol (CD_3OD 99.5% D) was from Bio-Rad labs. (Richmond, Calif.) or Wilmad Glass Co. Inc. (Buena, N.J.). Other materials were reagent grade and used without purification.

Methods. Proton magnetic resonance (pmr) spectra were recorded on a Varian HA-100 nuclear magnetic resonance (nmr) spectrometer operating in the frequency sweep mode at an ambient probe temperature between 34 and 35°. Tetramethylsilane was used as an internal lock standard. Chemical shifts were measured relative to tetramethylsilane by side band modulation techniques. All phosphatidylcholine[1] samples were dried over phosphorus pentoxide under vacuum (0.1 mm) at room temperature for 18–24 hr. Nmr sample tubes were flushed with dry nitrogen gas just prior to sample preparation. Infrared spectra were recorded on a Beckman IR-12 infrared spectrophotometer using variable path length cells with IR-TRAN windows from Precision Cells (Hicksville, N.Y.).

Results

Assignments for the various protons of phosphatidylcholine (I) in methanol have been previously reported (Birdsall *et al.*, 1972, Dufourcq and Lussan, 1972). Since many of the conclusions in this study were based on the interpretation of the pmr spectra of phosphatidylcholines, it is necessary to reiterate a few of the previously described results.

The pmr spectra of the glycerophosphorylcholine portion of 1,2-dibutyrylphosphatidylcholine in methanol is shown

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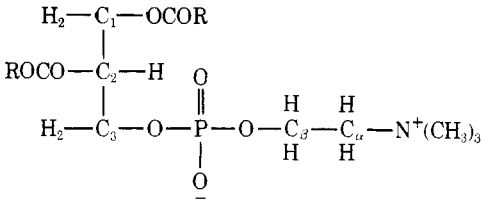
‡ Recipient of an NDEA Title IV Predoctoral Fellowship No. 71-00937.

[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 phosphatidylcholine (PC).



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in Figure 1A. The single low field proton multiplet at 5.31 ppm is assigned to the C_2 proton, and homonuclear spin decoupling experiments indicate that it is coupled to the C_1 and C_3 protons of the glycerol moiety. The C_1 protons give an octet at 4.35 ppm which corresponds to the AB part of an ABX (where X = C_2 proton) spin system with observed coupling constants J_{gem} = 12.0, J_{vic} = 7.0, 3.5 Hz. (The factoring of the peak is shown in Figure 1A.) These values agree closely with those reported by Birdsall *et al.* (1972) (J_{gem} = 11.9, J_{vic} = 7.0, 3.2 Hz) and Dufourcq and Lussan (1972) J_{gem} = 12.5, J_{vic} = 7.3, 3.25 Hz). The C_3 glycerol protons at 4.06 ppm are chemically equivalent giving the observed doublet of doublets corresponding to an A_2X (X = C_2 proton) spin system. Geminal coupling between the two protons is not observed. These protons are characterized by a ^{31}P-^1H spin coupling, $J_{P\text{-}H}$ = 6.8 Hz, clearly seen by homonuclear spin decoupling of the C_2 glycerol proton. The C_β choline protons give a broad resonance centered at 4.32 ppm. These protons are expected to give a complex multiplet from spin–spin interactions with vicinal protons, phosphorus, and nitrogen-14 neighbors (Birdsall *et al.*, 1972, Dufourcq and Lussan, 1972). Moreover, part of the C_1 glycerol proton resonances are superimposed on this resonance. The C_α choline protons at 3.68 ppm give a multiplet similar to that found in certain choline type analogs. This multiplet has been analyzed in terms of an AA′ part of an AA′ BB′ spin system (Birdsall *et al.*, 1972, Dufourcq and Lussan, 1972, Dufourcq *et al.*, 1972, Makriyannis *et al.*, 1972).

Figure 1B shows the same spectral region given in Figure 1A in the presence of 500 mM calcium chloride. The addition of calcium chloride to methanolic solutions of 1,2-dibutyrylphosphatidylcholine results in significant downfield shifts of all the protons of the glycerophosphorylcholine group, except those on C_1 and C_2 of the glycerol moiety. The protons of the acyl chains were not affected. There do not appear to be any changes in the vicinal coupling constants for the various protons, as shown for the C_1 and C_3 glycerol protons. The observed broadening of the peaks is probably due to viscosity effects.

The salt induced chemical shifts of the various protons as a function of calcium chloride concentration, using 100 mM dibutyrylphosphatidylcholine, are shown in Figure 2. The salt induced chemical shifts are nearly complete at 500 mM calcium. From previously reported data (Misiorowski and Wells, 1973) the formation of the phosphatidylcholine–calcium complex can be estimated to be more than 95% complete for the above phosphatidylcholine and calcium concentrations. The largest shifts are for the C_α and C_β choline protons with limiting shift values of 17 and 14 Hz, respectively. Somewhat smaller shift values are observed for the $N(CH_3)_3$ (9–10 Hz) and C_3 glycerol (7–8 Hz) protons.

Equilibrium 1 has been described for interaction of phosphatidylcholine (PC) and calcium in anhydrous methanol solutions (Misiorowski and Wells 1973). Under the condi-

$$PC + Ca = PC\text{-}Ca \quad (1)$$

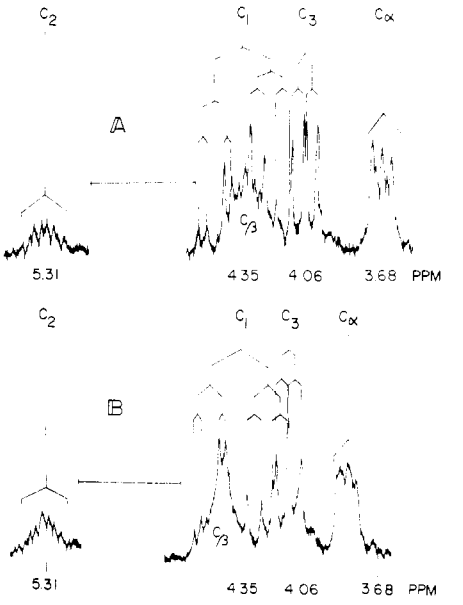

FIGURE 1: The partial 100-MHz pmr spectra of 100 mM dibutyrylphosphatidylcholine in methanol-d_4. (A) No calcium chloride added; (B) in the presence of 500 mM calcium chloride. The horizontal line width represents 50 Hz.

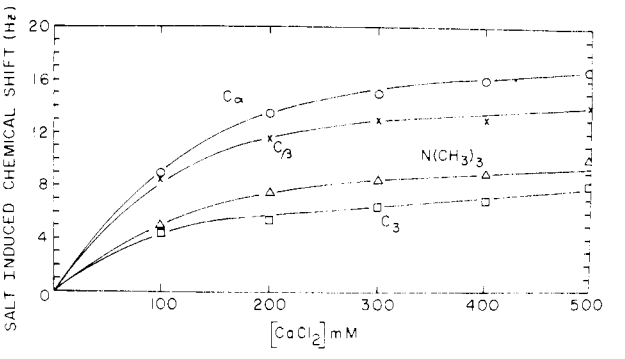

FIGURE 2: Calcium salt induced chemical shifts for the various protons of 100 mM dibutyrylphosphatidylcholine as a function of $CaCl_2$ concentration in methanol-d_4.

tions of rapid chemical exchange, the salt induced chemical shift observed for a given proton represents an average of the chemical shifts in the free and complexed environments. Following Prestegard and Chan (1969, 1970) we can describe the salt induced chemical shifts by

$$\delta = \frac{1}{2}\delta_c\{(1 + \phi + \eta) - [(1 + \phi + \eta)^2 - 4\phi]^{1/2}\} \quad (2)$$

where δ is the observed salt induced chemical shift, δ_c is the limiting chemical shift value of the proton in the complexed environment, ϕ is the stoichiometric concentration ratio of calcium to PC, and η is the reciprocal of the product of the apparent formation constant K and the stoichiometric PC concentration. By application of eq 2 and using the K_{ass} for PC–calcium complexes previously determined (Misiorowski and Wells 1973), theoretical binding curves for the equilibrium in eq 1 can be obtained. These curves are compared to the experimentally observed values of δ/δ_c and ϕ for two values of η in Figures 3A and B. The excellent agreement between the observed and theoretical data show that the salt induced chemical shifts are measuring the same equilibrium previously reported using equilbrium gel filtration

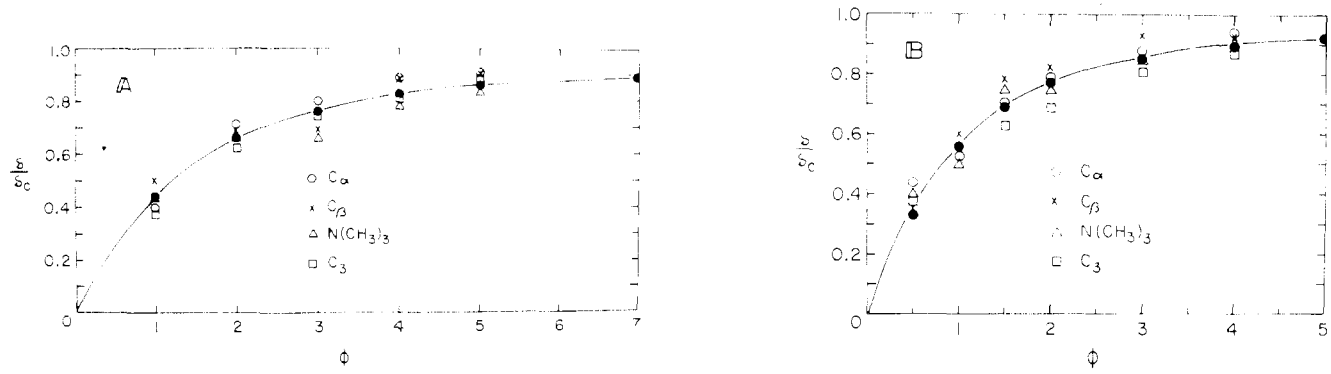

FIGURE 3: Experimental plots of δ/δ_c *vs* ϕ for dibutyrylphosphatidylcholines. Theoretical values of δ/δ_c as a function of ϕ are given by (●). (A) $\eta = 0.6992$; (B) $\eta = 0.3496$.

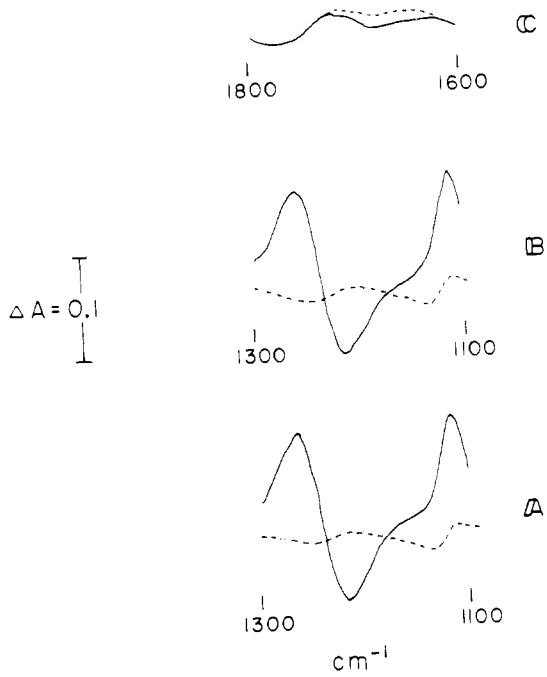

FIGURE 4: Infrared difference spectra of the P═O and C═O stretching regions of phosphatidylcholines in methanol in the presence of calcium chloride. (A) 100 mM *sn*-glycero-3-phosphorylcholine + 500 mM $CaCl_2$; (B and C) 100 mM hen's egg yolk phosphatidylcholines + 500 mM $CaCl_2$. The dotted lines represent the recorded base lines of 100 mM phosphatidylcholine in both sample and reference cells.

techniques (Misiorowski and Wells 1973), and support the conclusion that a 1:1 complex is formed. The calcium salt induced chemical shifts do not appear to be affected by the nature of the anion, since calcium bromide caused the same chemical shifts for the various protons described above at concentrations equal to that for calcium chloride.

In the presence of $MgCl_2$ similar salt induced chemical shifts were noted. Due to the hydroscopic nature of $MgCl_2$ and solutions containing $MgCl_2$, the spectra were complicated by the presence of water protons, and a detailed analysis was not carried out. However, sufficient data were collected to indicate that the relative chemical shifts were the same in the presence of $MgCl_2$ as reported above for $CaCl_2$. Furthermore, the apparent binding constant for Mg^{2+} to phosphatidylcholine was very similar to that observed for Ca^{2+}.

Further evidence for the interaction of phosphatidylcholine and calcium in anhydrous methanol solutions was obtained by infrared spectroscopy. Examination of the infrared spectrum of phosphatidylcholine in anhydrous methanol solutions in the presence of calcium showed a shift in the region 1150–1300 cm^{-1} which corresponds to the P═O stretching band. Differential infrared spectra of hen's egg yolk phosphatidylcholines in the presence and absence of calcium in anhydrous methanol showed a trough at approximately 1225 cm^{-1} and a maximum at 1265 cm^{-1}, as shown in Figure 4B. Thus the P═O stretching frequency is shifted to shorter wavelengths in the presence of calcium. It was possible that calcium could coordinate to the carbonyl oxygens of the acyl ester groups of phosphatidylcholine. However, no difference could be detected in the carbonyl stretching region (1700–1800 cm^{-1}) in the presence of calcium (Figure 4C). Further evidence to support the contention that the phosphoryl group was involved in phosphatidylcholine–calcium interactions in anhydrous methanol was supported by differential infrared spectra of *sn*-glycero-3-phosphorylcholine–calcium complexes which showed a trough at approximately 1215 cm^{-1} and a maximum at 1250 cm^{-1} (Figure 4A).

Lanthanide ions have been shown to interact with phosphatidylcholine vesicles in aqueous solutions (Andrews *et al.*, 1973; Bystrov *et al.*, 1971; Fernández and Cerbón, 1973) and with monomeric lecithins in methanol (Misiorowski and Wells, 1973). Although the K_{ass} for the formation of the lanthanide–phosphatidylcholine complex is large, these ions do not enhance the amine-catalyzed methanolysis of lecithin (Wells, 1974). It was anticipated that pmr studies might provide some information about the differences between the lanthanide–lecithin and calcium–lecithin complexes.

The results of these experiments are shown in Table I. The magnitude of the lanthanide induced chemical shifts was C_β choline > C_3 glycerol > C_α choline > $N(CH_3)_3$.

As in the case of calcium and magnesium the protons on C_1 and C_2 of glycerol were not appreciably shifted (less than 20 Hz for the cerium- and neodymium–dibutyryllecithin complexes and less than 70 Hz for the praseodymium–diacetyllecithin complex) at the highest lanthanide ion concentrations used. The protons on the acyl side chain were not shifted for the cerium- and neodymium–dibutyryllecithin complexes and less than 18 Hz for the praseodymium–diacetyllecithin complex at the highest lanthanide ion concentrations used. Changes in the vicinal coupling constants for the various protons in the lanthanide–phosphatidylcholine complexes could not be determined since resolution of the spin–spin splitting for the protons was lost. This is probably due to chemical exchange spin decoupling (Frankel, 1969).

TABLE I: Lanthanide Induced Isotropic Shifts of Protons in Phosphatidylcholine.[a]

Phosphatidylcholine and Lanthanide	Lanthanide (mM) 5	10	15	20	25	30
	Chemical Shift (Hz)					
(1) Diacetylphosphatidylcholine and praseodymium						
Choline C_β protons	53	110		201		290
C_3 glycerol protons	43	86		160		230
Choline C_α protons	22	44		70		90
Choline $N(CH_3)_3$ protons	8	12		15		
(2) Dibutyrylphosphatidylcholine and neodymium						
Choline C_β protons	29	69	94	110	130	
C_3 glycerol protons	25	48	70	78	95	
Choline C_α protons	13	27	36	39	42	
Choline $N(CH_3)_3$ protons					8	
(3) Dibutyrylphosphatidylcholine and cerium						
Choline C_β protons	33	68	90	114	128	
C_3 glycerol protons	24	47	61	89	93	
Choline C_α protons	15	27	35	39	41	
Choline $N(CH_3)_3$ protons					10	

[a] 100 mM phosphatidylcholine was used in all experiments. All shifts are downfield.

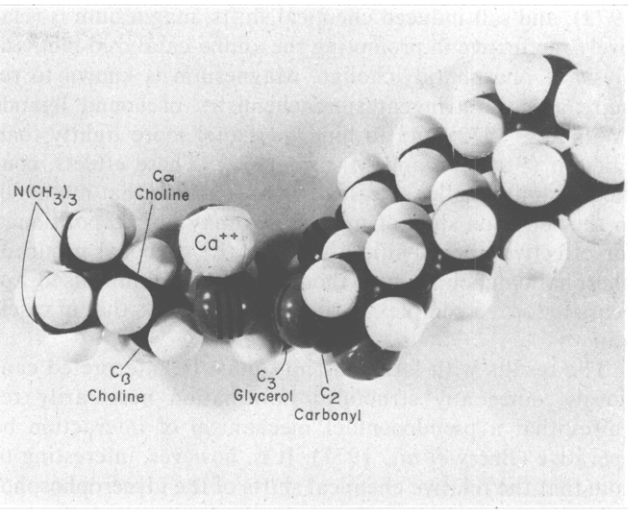

FIGURE 5: Space filling model of 1,2-diacyl-*sn*-glycero-3-phosphorylcholine showing the proposed site of calcium interaction. Calcium ion is denoted by the sphere.

Discussion

The data presented show that calcium and magnesium cause salt induced chemical shifts of specific protons of the lecithin molecule (I) in methanol solutions and suggest that the interaction of these ions is associated primarily with the phosphorylcholine moiety. In addition these results confirm the formation of a 1:1 complex between calcium or magnesium, and lecithin (Misiorowski and Wells, 1973). A proposed site of calcium or magnesium interaction with lecithin can be deduced from the following considerations.

The differences in the limiting chemical shifts can arise from any or all of the following effects. (1) The most straightforward effect is due to electrostatic polarization of the various C–H bonds by the bound divalent cation (Buckingham, 1960). This effect depends both on the distance between the cation and the proton and the relative orientation of the polarization field and the bond axis. (2) Cation induced conformational changes which change the relative orientation of polar substituents or magnetically anisotropic groups within the molecule (Prestegard and Chan, 1970). Although we cannot eliminate these effects entirely, the apparent unchanging values for the various coupling constants would tend to minimize these effects. (3) Differences in the polarization of the solvent surrounding the molecule in the presence and absence of the cation. We cannot estimate the magnitude of these effects, but have assumed that such effects will only be important when the cation is near a proton. Thus we consider that effects 1 and 3 are primarily important, and further assume that the sum of both effects is due to the proximity of the bound cation.

Infrared spectral studies show that the P=O stretching band of lecithin shifts to shorter wavelength in the presence of calcium. No effects were noted on the carbonyl stretching band in the presence of calcium. Taken with the magnitude of the salt induced chemical shifts, *i.e.*, $C_\alpha > C_\beta > N(CH_3)_3 > C_3$, and the lack of effect of calcium on the protons at C_1, C_2, and the acyl chain protons, we feel the structure shown in Figure 5 represents a reasonable proposal for the calcium–lecithin complex.

The conformation about the C–C bonds of the C_2–C_1, C_2–C_3 glycerol and C_β–C_α choline fragments are fixed to be consistent to one of the average rotamer populations deduced from the observed vicinal coupling constant analyses (Birdsall *et al.*, 1972, Dufourcq and Lussan 1972). The relative distance from the center of the calcium (denoted by the sphere in Figure 5) to the various centers of the carbon atoms bearing the protons under consideration were $C_3 > N(CH_3)_3 > C_\beta > C_\alpha$. In the proposed structure calcium is placed between the two phosphate oxygens, which are equivalent by resonance.

The structure proposed is considered to be the most probable for the following reasons. (1) Rotation of the H_2C_3 glycerol group ±180° about the C_2–C_3 bond results in the other possible average rotamer population based on vicinal coupling constant analyses. If calcium is placed between the two phosphoryl oxygens in this structure, it appears that significant perturbations of the diamagnetic shielding of the C_2 and C_1 glycerol protons would be expected. Since these protons are not influenced by calcium, we consider this structure unlikely. (2) Rotating the phosphorus atom ±180° about the C_3–O–P bond places the protons on the C_3 glycerol group too close to the calcium to be consistent with the observed magnitude of their chemical shift. (3) If we consider the role of calcium in the methanolysis of phosphatidylcholine (Wells, 1974), then the structure shown in Figure 5 again appears most probable. Methanol in the solvation shell of calcium lies quite close to the carbonyl groups at C_1 and C_2 in the proposed structure. If either rotation described above is performed, then the methanol in the solvation shell of calcium is too far removed from the carbonyl groups to be considered important in the methanolysis reaction.

Although we have discussed the structure of the metal ion complex in terms of Ca^{2+}, a similar analysis would also apply to the magnesium complex. However, in spite of a similar binding constant; inhibitory effect on phospholipase A_2 in methanol–water solutions (Misiorowski and Wells,

1973); and salt induced chemical shifts, magnesium is relatively ineffective in promoting the amine-catalyzed methanolysis of phosphatidylcholine. Magnesium is known to require a more stringent stereochemistry of bound ligands (Williams, 1972) and to bind methanol more tightly than calcium (Stockton and Martin, 1972). These effects, coupled with its smaller size, lead us to conclude that methanol in the solvation shell of magnesium may not be positioned for effective nucleophilic attack on the carbonyl group of phosphatidylcholine, even though the magnesium itself appears to form a complex of similar structure to that of calcium.

The results with lathanide ions must be interpreted cautiously, since any structural information necessarily requires that a pseudocontact mechanism of interaction be operative (Barry *et al.*, 1971). It is, however, interesting to note that the relative chemical shifts of the glycerophosphorylcholine protons is different in the presence of the lanthanide ions than in the presence of calcium or magnesium. These results may suggest that the structure of the lathanide–lecithin complex is different than the calcium–lecithin complex. Such a structural difference would also be consistent with the inability of lanthanide ions to enhance the amine catalyzed methanolysis of lecithin (Wells, 1974).

The results of these experiments suggest a probable site of calcium binding to phosphatidylcholines. The interaction of calcium and lecithins as shown in Figure 5 is consistent with the pmr and infrared spectroscopic data. The results of this study also shed further insight into the binding of cations to phosphatidylcholines in methanol (Misiorowski and Wells, 1973) and other organic solvents, and provide a partial explanation of the high specificity for calcium in enhancing the amine-catalyzed methanolysis of lecithins (Wells, 1974). It also appears that both the structure and reactivity of the cation–lecithin complex are dependent on the nature of the cation.

Acknowledgments

We thank the Department of Chemistry for the use of the Varian HA-100 nmr spectrometer.

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