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Stabilities of Metal Complexes of Phospholipids: Ca(II), Mg(II), and Ni(II) Complexes of Phosphatidylserine and Triphosphoinositide*

H. Stewart Hendrickson and J. G. Fullington

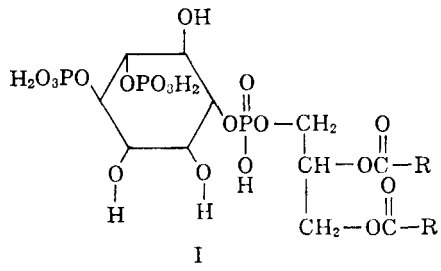
ABSTRACT: Stability constants for Ca(II), Mg(II), and Ni(II) complexes of phosphatidylserine, triphosphoinositide, *O*-phosphoserine, *O*-phosphoethanolamine, and glycerylphosphorylinositol diphosphate were determined by a *p*H titration method. The lipids were studied in aqueous micellar dispersions. Apparent stability constants (K^{M}_{ML} and K^{M}_{MHL}) for the intact lipids were 10 to 100 times greater than those for the deacylated models. These constants include a free-energy factor due to the electrostatic field of the micelle surface and are not a true measure of complexing ability. Displacement constants, which represent the equilibrium $H_2L + M^{2+} \rightleftharpoons ML + 2\ H^+$, are a more valid measure of complexing ability since this reaction involves no net change in charge on the ligand and the electrostatic free energy terms disappear. Comparison of displacement constants indicates that triphosphoinositide and glycerylphosphorylinositol diphosphate differ very little in complexing ability. Similar comparisons with phosphatidylserine and phosphoserine indicate greater complexing ability for the lipid. Phosphoserine, however, may not be a suitable model for comparison with phosphatidylserine. The former is a phosphate monoester and the latter a diester.

The binding of alkali and alkaline earth metals by phosphatides was suggested by some of the earliest investigators of lipid chemistry (Thudichum, 1901; Koch and Pike, 1910; Koch and Todd, 1911). The first quantitative study of sodium and potassium binding by cephalin was reported by Christensen and Hastings (1940). Since then very little work has been reported on phospholipid metal binding. Recently, however, many investigators have again emphasized the importance of this type of metal binding in biological systems. There are indications that such metal chelation is quite important in lipoprotein formation, cation transport, and other biochemical processes. Abramson *et al.* (1964) reported studies on the ionic properties of phosphatidylserine which indicate strong Ca(II) binding by that lipid. Hakamori *et al.* (1963) reported the isolation of a metal-bound lipid-peptide complex from ox brain, and Carter *et al.* (1962) postulated that phytoglycolipid occurs in plants as a mixed Ca(II) and Mg(II) chelate. Ca(II) and Mg(II) dramatically affect the solubilities and ionic properties of brain phosphoinositides (Hendrickson and Ballou, 1964). Papahadjopoulos and Hanahan (1964) demonstrated the requirement of

* From the Western Regional Research Laboratory (a laboratory of the Western Utilization Research and Development Division), Agricultural Research Service, U.S. Department of Agriculture, Albany, Calif. *Received March 4, 1965.* Presented in part at the 148th National Meeting of the American Chemical Society, Chicago, Ill., September 1964.

Ca(II) in the formation of a lipoprotein complex exhibiting full activity as a prothrombin activator.

This investigation was begun to study the metal-chelating properties of phospholipids and the possibility of mixed chelation as a type of interaction in wheat flour lipoproteins. Stabilities of the Ca(II), Mg(II), and Ni(II) complexes of triphosphoinositide (structure I) and phosphatidylserine (Figure 8) are reported here. Metal complexes of deacylated triphosphoinositide, *O*-phosphoserine, and *O*-phosphoethanolamine were also studied to compare the effects of micelle formation and of different ionic groups on metal complex stability. The two alkaline earth metals were chosen because many phospholipids are isolated from their natural sources as salts of these metals. Ni(II) was chosen to compare the binding of a transition series metal with the binding of alkaline earth metals and also because many data are available on the stabilities of Ni(II) complexes of biologically important compounds (Hammes and Morrell, 1964; Lenz and Martell, 1964a,b).



I

Experimental

Routine Analytical Methods. Phosphorus was determined by the method of Bartlett (1959), and fatty acid esters according to Rapport and Alonzo (1955). The purity of the lipids was further checked by thin-layer silicic acid chromatography of the intact lipids and paper chromatography of the deacylated lipids as described by Hendrickson and Ballou (1964).

Isolation and Purification of Ligands. Phosphatidylserine and triphosphoinositide were obtained by Folch fractionation of beef brain (Folch, 1949; Lees, 1957). The crude lipids were further purified by chromatography on DEAE-cellulose as described by Hendrickson and Ballou (1964). The lipids were pure as shown by the expected ester-to-phosphorus ratios, their migration on thin-layer silicic acid, and paper chromatography of the deacylated lipids. Since the lipids were isolated as ammonium salts, they were converted to the lithium salts for titration studies by passage through Dowex 50-X8 (Li^+, 200–400 mesh) resin. In some cases, the lipids were converted directly to the acidic form by passage through Dowex 50-X8 (H^+, 200–400 mesh) resin. The lipids were titrated immediately to avoid any acid autohydrolysis.

Triphosphoinositide was soluble in water. At the concentrations used, micelles formed which were not retained on G-200 Sephadex. Aqueous micellar dispersions of phosphatidylserine were prepared by sonication of a 5–10% suspension at 10 kc under a nitrogen atmosphere for 10 minutes (Abramson *et al.*, 1964). The ester-to-phosphorus ratio was unchanged after this treatment and the sonicated dispersions were clear and colorless, and stable for several weeks when stored at 4°.

Glycerylphosphorylinositol diphosphate was prepared by deacylation of triphosphoinositide and purified by chromatography on Dowex 1 resin as described by Hendrickson and Ballou (1964).

DL-*O*-Phosphoserine was obtained from California Corp. for Biochemical Research and *O*-phosphoethanolamine from Mann Research Laboratories. These compounds were quite pure and were used without further treatment.

Other Reagents. Stock 0.2 M Mg(II), Ca(II), and Ni(II) solutions were prepared from the analytical grade chlorides or nitrate in the case of Ni(II). The solutions were standardized by the EDTA titration procedures of Schwarzenbach (1956). Tetrapropylammonium iodide and tetrapropylammonium hydroxide (10% in water) were obtained from Eastman Organic Chemicals.

*p*H *Titrations.* Titration curves were obtained with a Radiometer[1] automatic titrator equipped with glass and calomel electrodes. The temperature of the titration vessel was maintained at 20° and nitrogen gas was bubbled through the solution to prevent air oxidation and CO_2 absorption. The *p*H meter was calibrated with buffers of appropriate *p*H.

A measured amount of ligand (5–20 μmoles, based on phosphorus analysis) was pipetted into the vessel followed by 4 ml of 0.25 M tetrapropylammonium iodide[2] and sufficient 0.1 N HCl to bring the starting *p*H to about 3–3.5. A measured amount of metal was then added as required and the final volume adjusted to 10 ml. Titration was begun with 0.05 or 0.10 M tetrapropylammonium hydroxide as the titrant. The titration curves were recorded automatically (at the rate of 0.5 *p*H unit/min) and manually (checked for complete equilibration at each point). Titration curves obtained by these two procedures were identical within an experimental error of ±0.05 *p*H unit. Reverse titrations indicated no irreversible changes. All titration curves were corrected for the contribution from water by subtracting a titration curve for water obtained under identical conditions. The stoichiometry determined independently (by phosphorus analysis) agreed with that determined from the titration curves, indicating that all acidic groups were available for titration.

The titration curves are shown in Figures 1–5. The ordinates refer to *p*H meter readings and represent hydrogen ion activities. For subsequent calculations, these activities were converted to concentrations by the

[1] Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

[2] At high metal concentrations, less tetrapropylammonium iodide was added in order to maintain the ionic strength at 0.1 M.

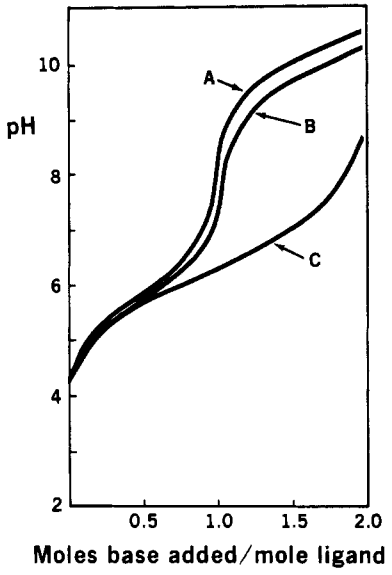


FIGURE 1: Titration curves for *O*-phosphoserine. A, Free ligand; B, Mg(II)-ligand (5:1); C, Ni(II)-ligand (1:1) ($C_0 = 5.10 \times 10^{-4}$ M, $\mu = 0.1$ M).

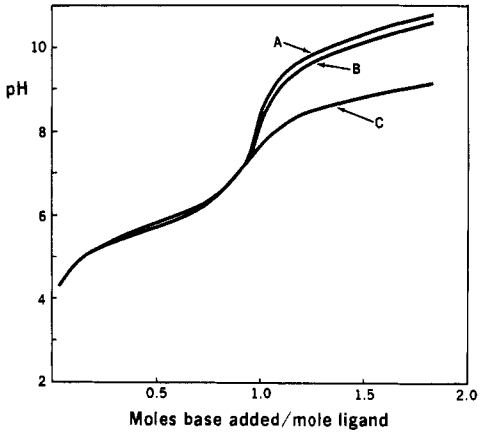


FIGURE 2: Titration curves for *O*-phosphoethanolamine. A, Free ligand; B, Mg(II)-ligand (5:1); C, Ni(II)-ligand (1:1) ($C_0 = 2.01 \times 10^{-3}$ M, $\mu = 0.1$ M).

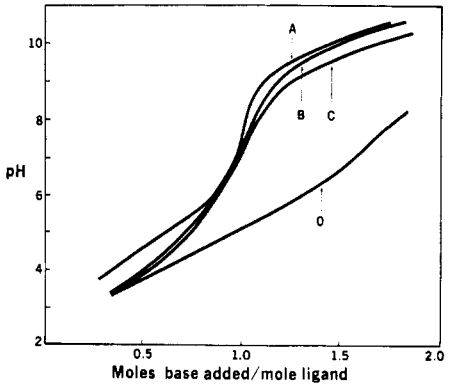


FIGURE 3: Titration curves for phosphatidylserine. A, Free ligand; B, Ca(II)-ligand (1:1); C, Mg(II)-ligand (1:1); D, Ni(II)-ligand (1:1) ($C_0 = 1.05 \times 10^{-3}$ M, $\mu = 0.1$ M).

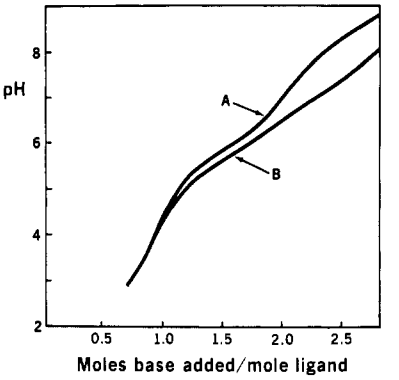


FIGURE 4: Titration curves for glycerylphosphorylinositol diphosphate. A, Free ligand; B, Mg(II)-ligand (5:1) ($C_0 = 4.73 \times 10^{-4}$ M, $\mu = 0.1$ M).

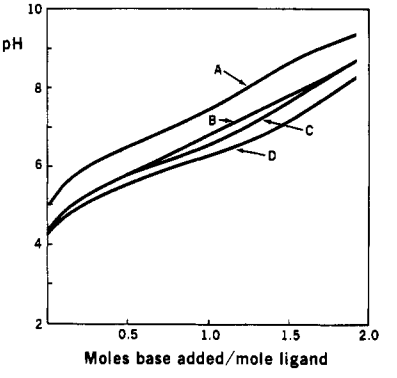


FIGURE 5: Titration curves for triphosphoinositide. A, Free ligand; B, Mg(II)-ligand (1:1); C, Ca(II)-ligand (1:1); D, Ni(II)-ligand (1:1) ($C_0 = 8.26 \times 10^{-4}$ M, $\mu = 0.1$ M).

relationship $[H^+] = a_{H^+}/\gamma_{H^+}$, assuming an activity coefficient $\gamma_{H^+} = 0.799$ for the hydrogen ion at 20° and 0.1 M ionic strength (Kortüm and Bockris, 1951).

Calculations and Results

In calculating the stability constants for the simple, nonlipid metal complexes, the following equilibria and equilibrium constants were considered

$$\begin{array}{ccccc} & & K_{A2} & & K_{A1} \\ & H_2L & \rightleftharpoons & HL^- & \rightleftharpoons & L^{2-} \\ & K^{M}_{MHL}\uparrow\downarrow & & & & \downarrow\uparrow K^{M}_{ML} \\ & MHL^+ & \rightleftharpoons & ML & & \end{array}$$

$$K_{A1} = \frac{[L^{2-}][H^+]}{[HL^-]}; \qquad K_{A2} = \frac{[HL^-][H^+]}{[H_2L]} \tag{1}$$

$$K^{M}_{ML} = \frac{[ML]}{[M^{2+}][L^{2-}]}; \qquad K^{M}_{MHL} = \frac{[MHL^+]}{[M^{2+}][HL^-]} \tag{2}$$

where H_2L = protonated ligand.

Using the calculations of Hammes and Morrell (1964), two types of equations can be derived. An equation for the free metal concentration can be derived in the form

$$[M^{2+}] = \frac{-B + \sqrt{B^2 + 4AC}}{2A} \quad (3)$$

where

$$A = \frac{[H^+]K^M_{MHL}}{K_{A1}} + K^M_{ML}$$

$$B = 1 + \frac{[H^+]}{K_{A1}} + C_0K^M_{ML} + \frac{C_0K^M_{MHL}[H^+]}{K_{A1}} + \frac{[H^+]^2}{K_{A1}K_{A2}} - \frac{C_MK^M_{MHL}[H^+]}{K_{A1}} - C_MK^M_{ML}$$

$$C = \frac{C_M[H^+]}{K_{A1}} + C_M + \frac{C_M[H^+]^2}{K_{A1}K_{A2}}$$

and C_M = total metal concentration, C_0 = total ligand concentration, $[H^+]$ = hydrogen ion concentration in the solution. Two simultaneous equations representing two different points on the curve can be derived in the form

$$\begin{aligned} a_1K^M_{ML}[M^{2+}] + b_1K^M_{MHL}[M^{2+}] &= C_1 \\ a_2K^M_{ML}[M^{2+}] + b_2K^M_{MHL}[M^{2+}] &= C_2 \end{aligned} \quad (4)$$

where

$$a = C_0 - [OH^-]_{add} + [H^+]_* - [H^+]$$

$$b = \frac{K_{A1}}{[H^+]}(2C_0 - [OH^-]_{add} + [H^+]_* - [H^+])$$

$$C = ([OH^-]_{add} - 2C_0 - [H^+]_* + [H^+]) \times \left(1 + \frac{K_{A1}}{[H^+]} + \frac{[H^+]}{K_{A2}}\right) + C_0 + 2C_0\frac{[H^+]}{K_{A2}}$$

and $[H^+]_*$ = hydrogen ion concentration at point where $[L^{2-}] = 100\%$, $[OH^-]_{add}$ = concentration of hydroxide ion added starting from the point where $[H_2L] = 100\%$.

Using values for $[H^+]_*$, $[OH^-]_{add}$, and $[H^+]$ from two different points on the titration curve along with approximate values of $[M^{2+}]$ in equation (4), values for K^M_{ML} and K^M_{MHL} can be determined.

Using these values for K^M_{ML} and K^M_{MHL} in equation (3), a second approximation of $[M^{2+}]$ can be obtained. By repeating this process until the values for $[M^{2+}]$ used in the two equations are equal, true values for K^M_{ML} and K^M_{MHL} can be determined.

Ionization and chelate stability constants for polymeric ligands, such as lipid micelles, vary with the degree of ionization because of the changing electrostatic field of the micelle surface (Rice and Nagasawa, 1961). For a polymeric acid, the ionization constant varies with the electrostatic free energy in the following manner

$$K_A = K_0e^{-\Delta Fe/kT} \quad (5)$$

where K_A is the apparent ionization constant, K_0 is the intrinsic ionization constant, and ΔFe is the electrostatic free-energy change for the ionization (Bjerrum, 1923). Katchalsky and Spitnik (1947) have proposed the following modified Henderson-Hasselbalch equation to describe the ionization of a polyelectrolyte

$$pH = pK_A - n \log [(1 - \alpha)/\alpha] \quad (6)$$

where α is the degree of ionization, n is a constant, and K_A is the apparent ionization constant. K_A and n are independent of α for a constant ionic strength and polymer concentration. K_A increases, and n decreases with increasing ionic strength. For simple monomeric acids $n = 1$, but for polymeric acids $n > 1$.

Due to the electrostatic free-energy factor, apparent stability constants (K^M_{ML} and K^M_{MHL}) for the lipid metal complexes cannot be compared directly with those for the simple models. Gregor *et al.* (1955) proposed a "displacement constant" (B) which can be used to compare binding of polymeric and monomeric ligands. B is defined by the equilibrium

$$M^{2+} + H_2L \rightleftharpoons ML + 2H^+$$

where

$$B = \frac{[ML][H^+]^2}{[M^{2+}][H_2L]} = K_{A1}K_{A2}K^M_{ML} \quad (7)$$

This reaction involves no net change in charge on the ligand, and these equilibrium constants can be compared directly for monomeric and polymeric ligands. In this treatment it is assumed that the electrostatic free-energy changes (ΔFe) for the dissociation of two protons and for the association of a divalent metal ion are equal and opposite. Thus, the two electrostatic free-energy terms disappear, and B is a more valid measure of the complexing ability of the ligand.

Apparent ionization constants K_{A1} and K_{A2} for the lipids were calculated from equation (6). Plots of $-\log [H^+]$ versus $\log [(1 - \alpha)/\alpha]$ for triphosphoinositide, phosphatidylserine, and their analogous models, in the absence of metal ions, are shown in Figures 6 and 7. For the simple models, straight lines are obtained with $n = 1$, as expected. Triphosphoinositide also gives straight lines with $n = 1$ for both ionizations. This indicates very little change in the electrostatic free-energy term [$e^{-\Delta Fe/kT}$, equation (5)] going from H_2L^{3-} to HL^{4-} and HL^{4-} to L^{5-} since the micelle has a large negative charge initially. For phosphatidylserine, ionizations of the carboxyl group (K_{A2}) and the amino group (K_{A1}) both give straight lines with $n = 1.8$ and $n = 1.2$, respectively. This indicates a large change in the electrostatic free energy term going from H_2L to HL^{1-} and a smaller change going from HL^{1-} to L^{2-}.

Apparent stability constants (K^M_{ML} and K^M_{MHL}) for the metal-lipid complexes were calculated in the same man-

TABLE I: Equilibrium Stability Constants.[a]

Cation (metal:ligand)	Log K^{M}_{ML} (M^{-1})	Log K^{M}_{MHL} (M^{-1})	Log B (M)
	O-Phosphoserine		
H^+	9.90 ± 0.05	5.80 ± 0.05	
Mg(II) (5:1)	3.28 ± 0.1	2.57 ± 0.1	−12.4 ± 0.1
Mg(II) (10:1)	3.24 ± 0.1	2.45 ± 0.1	−12.5 ± 0.1
Ca(II) (5:1)	3.30 ± 0.1	2.57 ± 0.1	−12.4 ± 0.1
Ca(II) (10:1)	3.18 ± 0.1	2.40 ± 0.1	−12.5 ± 0.1
Ni(II) (1:1)	6.69 ± 0.1	2.65 ± 0.1	−9.0 ± 0.1
Ni(II) (2:1)	6.69 ± 0.1	2.63 ± 0.1	−9.0 ± 0.1
	O-Phosphoethanolamine		
H^+	10.26 ± 0.05	5.77 ± 0.05	
Mg(II) (5:1)	2.15 ± 0.1	1.48 ± 0.1	−13.9 ± 0.1
Mg(II) (10:1)	2.20 ± 0.1	1.42 ± 0.1	−13.8 ± 0.1
Ca(II) (5:1)	2.06 ± 0.1	1.49 ± 0.1	−14.0 ± 0.1
Ca(II) (10:1)	1.92 ± 0.1	1.19 ± 0.1	−14.0 ± 0.1
Ni(II) (1:1)	4.66 ± 0.1	1.97 ± 0.1	−11.4 ± 0.1
Ni(II) (2:1)	4.56 ± 0.1	1.64 ± 0.1	−11.5 ± 0.1
	Phosphatidylserine[b]		
H^+	9.93 ± 0.05	4.42 ± 0.05	
Mg(II) (1:1)	4.34 ± 0.1	3.91 ± 0.1	−10.1 ± 0.1
Mg(II) (2:1)	4.21 ± 0.1	3.74 ± 0.1	−10.1 ± 0.1
Ca(II) (1:1)	4.13 ± 0.1	4.03 ± 0.1	−10.2 ± 0.1
Ni(II) (1:1)	7.94 ± 0.1	4.56 ± 0.1	−6.4 ± 0.1
	Glycerylphosphorylinositol diphosphate		
H^+	8.05 ± 0.05	5.70 ± 0.05	
Mg(II) (5:1)	3.45 ± 0.1	2.37 ± 0.1	−10.3 ± 0.1
Mg(II) (10:1)	3.47 ± 0.1	2.31 ± 0.1	−10.3 ± 0.1
Ca(II) (5:1)	3.27 ± 0.1	2.22 ± 0.1	−10.5 ± 0.1
	Triphosphoinositide[b]		
H^+	8.45 ± 0.05	6.38 ± 0.05	
Mg(II) (1:1)	4.85 ± 0.1	3.76 ± 0.1	−10.0 ± 0.1
Mg(II) (2:1)	5.30 ± 0.1	3.76 ± 0.1	−9.5 ± 0.1
Ca(II) (1:1)	5.04 ± 0.1	3.83 ± 0.1	−9.8 ± 0.1
Ni(II) (1:1)	5.92 ± 0.1	4.35 ± 0.1	−8.9 ± 0.1
Ni(II) (2:1)	6.67 ± 0.1	4.03 ± 0.1	−8.2 ± 0.1

[a] T = 20°, μ = 0.1 M tetrapropylammonium iodide. [b] For lipid metal complexes, K^{M}_{ML} and K^{M}_{MHL} are apparent stability constants at the points of half-ionization (α = 0.5).

ner as for the simple models, using titration data from the points of half-ionization (α = 0.5) only. Using equation (7), displacement constants (B) were calculated. The stability and displacement constants are given in Table I.

Discussion

The stability constants reported here were all calculated assuming a 1:1 metal-to-ligand complex formed. This assumption seems valid since constants calculated at two different metal concentrations are identical within experimental error with the exception of K^{M}_{MHL} for Ca(II)- and Ni(II)-phosphoethanolamine, and K^{M}_{ML} for Mg(II)- and Ni(II)-triphosphoinositide. The former constants could reflect the formation of a 1:2 metal-phosphoethanolamine complex at lower metal concentrations. The greater apparent K^{M}_{ML} values for Mg(II)- and Ni(II)-triphosphoinositide at higher metal concentrations could reflect the formation of a 2:1 metal-ligand complex.

Comparison of metal binding by phosphoserine with that by other ligands that lack one or more of the functional groups (Table II) indicates that phosphoserine (and presumably phosphatidylserine) acts as a tridentate ligand with the metals studied here. The phosphoserine-Mg(II) complex is about ten times more stable than the phosphoethanolamine or alanine complexes. This

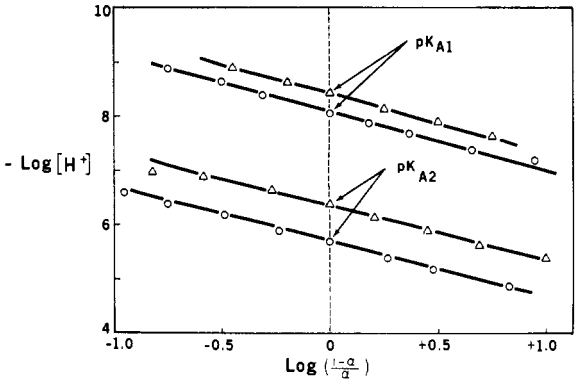


FIGURE 6: Plots of $-\log [H^+]$ versus $\log ([1 - \alpha]/\alpha)$ for triphosphoinositide (Δ) ($C_0 = 8.26 \times 10^{-4}$ M, $\mu = 0.1$ M) and glycerylphosphorylinositol diphosphate (O) ($C_0 = 4.73 \times 10^{-4}$ M, $\mu = 0.1$ M).

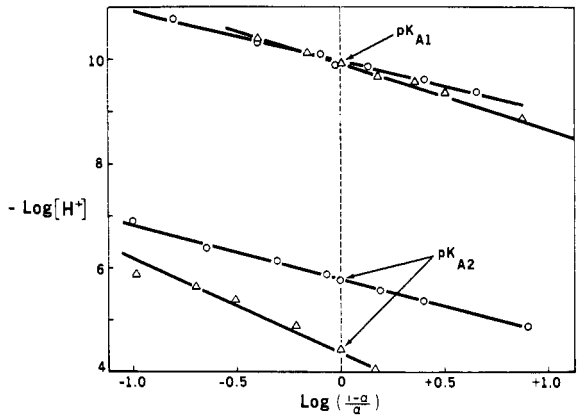


FIGURE 7: Plots of $-\log [H^+]$ versus $\log ([1 - \alpha]/\alpha)$ for phosphatidylserine (Δ) ($C_0 = 1.05 \times 10^{-3}$ M, $\mu = 0.1$ M) and *O*-phosphoserine (O) ($C_0 = 5.10 \times 10^{-4}$ M, $\mu = 0.1$ M).

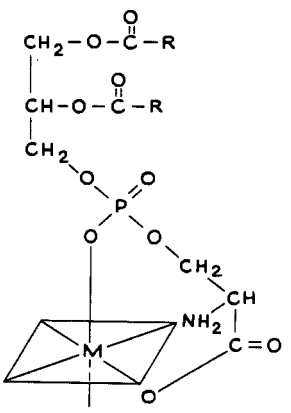


FIGURE 8: Possible structure for phosphatidylserine metal complex.

TABLE II: Comparison of Metal Chelate Stabilities of Phosphoserine and Related Compounds Lacking One or More Functional Groups.

Compound	Mg(II) Log K^M_{ML} (M^{-1})	Ni(II) Log K^M_{ML} (M^{-1})
O-Phosphoserine	3.28[a]	6.69[a]
O-Phosphoethanolamine	2.20[a]	4.66[a]
Alanine	2.00[b]	5.93[b]
HPO_4^{2-}	1.88[c]	

[a] This investigation ($T = 20°$, $\mu = 0.1$ M). [b] Monk (1951) ($T = 25°$, $\mu = 0.001$ M). [c] Smith and Alberty (1956) ($T = 25°$, $\mu = 0.2$ M).

increased stability must reflect participation by all three donor groups. Thus, a possible structure for the phosphatidylserine metal complex is that shown in Figure 8. Three of the six metal coordination bonds are free to accept additional donor groups. Mixed chelates could then be formed with other ligands such as amino acids or proteins. This type of structure could account for the metal-dependent lipoprotein interactions observed by Papahadjopoulos and Hanahan (1964) and Hakamori *et al.* (1963).

Glycerylphosphorylinositol diphosphate and triphosphoinositide form 1:1 metal-ligand complexes, although the intact lipid seems to complex with additional metal ions at higher metal concentrations. Molecular models of triphosphoinositide indicate that the most probable site for metal chelation is between the two monoesterified phosphate groups. Further studies are planned to establish the exact structure of this complex.

Ni(II) forms more stable complexes than the alkaline earth metals, especially with ligands containing a nitrogen donor, as would be expected for a transition metal (Keller and Parry, 1956). There is not much difference in the stabilities of Ca(II) and Mg(II) complexes of a given ligand.

Consideration of the micellar structure of lipids is quite important in the understanding of lipid-metal complexing. Abramson *et al.* reported studies of ultrasonicated phosphatidylserine dispersions in water. Their dispersions consisted of micelles with an average molecular weight of 4×10^6 (about 4800 molecules/micelle). All of the ionizable groups were shown to be on the surface since they were all readily accessible to titration. Triphosphoinositide is readily soluble in water and forms micelles of undetermined size.[3]

The apparent stability constants (K^M_{ML} and K^M_{MHL}) for the lipid metal complexes are much greater than those for the simple model complexes. However, these

[3] The molecular weight is greater than 2×10^5 since the micelles are not retained on G-200 Sephadex.

apparent stability constants are not a true measure of complexing ability since they include a free-energy factor due to the electrostatic field of the micelle surface. A more valid measure of complexing ability is the displacement constant (*B*) [see equation (7)]. The displacement constants for triphosphoinositide and glycerylphosphorylinositol diphosphate metal complexes are not significantly different, indicating that these ligands differ very little in complexing ability. The displacement constants for the phosphatidylserine metal complexes, however, are significantly greater than those for the phosphoserine metal complexes. They indicate a greater complexing ability for the lipid micelle. However, phosphoserine may not be a suitable model to compare with phosphatidylserine, the former being a phosphate monoester and the latter a diester. Comparison with a suitable phosphate diester model would clarify this point. Indeed, much more study is needed on the nature of metal complexing with phosphate esters in order to understand better the role of these compounds in biochemical processes.

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