

difference in eq A1 between the two phases:

$$0 = -\Delta\tilde{S}_m d\tau_m - \Delta\alpha_m d\tilde{T}$$

or

$$\left(\frac{\partial\tilde{T}}{\partial\tau_m}\right) = -\frac{\Delta\tilde{S}_m}{\Delta\alpha_m}$$

where the variations are taken along the locus of transitions; $\Delta\tilde{S}_m$ is the difference in entropy density between the two phases and for a reversible process is equal to the heat of melting per unit area divided by the melting temperature, τ_m ; $\Delta\alpha_m$ is the fractional change in surface area between the liquid and solid phases. Therefore

$$\left(\frac{\partial\tilde{T}}{\partial\tau_m}\right) = -\frac{Q_m}{\tau_m\Delta\alpha_m}$$

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Phosphorus-31 and Carbon-13 Nuclear Magnetic Resonance Studies of Divalent Cation Binding to Phosphatidylserine Membranes: Use of Cobalt as a Paramagnetic Probe[†]

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ABSTRACT: The paramagnetic divalent cation cobalt has large and well-understood effects on NMR signals from ligands bound in the first coordination sphere, i.e., inner-sphere ligands, and we have used these effects to identify divalent cation binding sites at the surface of phosphatidylserine membranes. ³¹P NMR results show that 13% of the bound cobalt ions are involved in inner-sphere complexes with the phosphodiester group, while ¹³C NMR results show that 54% of the bound cobalt ions are involved in unidentate inner sphere complexes with the carboxyl group. No evidence is found for cobalt binding to the carbonyl groups, but proton release studies

suggest that 32% of the bound cobalt ions are involved in chelate complexes that contain both the carboxyl and the amine groups. All (i.e., 13% + 54% + 32% = 99%) of the bound cobalt ions can thus be accounted for in terms of inner sphere complexes with the phosphodiester group or the carboxyl group. We suggest that the unidentate inner-sphere complex between cobalt and the carboxyl group of phosphatidylserine and the inner-sphere complex between cobalt and the phosphodiester group of phosphatidylserine provide reasonable models for complexes between alkaline earth cations and phosphatidylserine membranes.

Thermodynamic aspects of the interaction between calcium and bilayer membranes containing phosphatidylserine can be explained in terms of the Gouy-Chapman-Stern theory (McLaughlin et al., 1981), but the functional groups that form ligands for calcium have not been identified. Some information has been obtained from the effects of calcium on the NMR signals from phosphatidylserine membranes (Kurland et al., 1979a,b), but these effects are small and difficult to interpret theoretically because calcium is diamagnetic. In contrast,

paramagnetic divalent transition metal cations have large and well-understood effects on the NMR signals from ligands bound in the first coordination sphere, i.e., inner-sphere ligands (Shulman et al., 1965; McDonald & Phillips, 1963; Strouse & Matwiyoff, 1970; Swift & Connick, 1962) and can be used as "probes" for the divalent cation binding site at the surface of phospholipid membranes. Cobalt is the most useful paramagnetic divalent cation for this purpose because its effects on the NMR signals of inner-sphere ligands are easiest to quantitate (Shulman et al., 1965).

Sonicated phospholipid vesicles show "high-resolution" ³¹P NMR spectra (Borden et al., 1974) and ¹³C NMR spectra (Metcalfe et al., 1971; Batchelor et al., 1972), and distinct signals can be observed from most of the potential ligands in

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the polar head-group region. The effects of cobalt on the ^{31}P NMR and ^{13}C NMR spectra of phospholipid membranes can thus be used to identify the functional groups that form inner-sphere complexes with cobalt. We have previously demonstrated that only the phosphodiester group in phosphatidylglycerol membranes forms inner-sphere complexes with cobalt (Lau et al., 1981). In this paper we show that both the phosphodiester group and the carboxyl group in phosphatidylserine membranes form inner-sphere complexes with cobalt, and we discuss the relevance of these results to the structure of the complexes formed between alkaline earth cations and phosphatidylserine.

Materials and Methods

Materials. Bovine brain phosphatidylserine (PS) was purchased from Avanti Biochemicals (Birmingham, AL). In the final step of their purification procedure, it was partitioned against 0.5% ethylenediaminetetraacetic acid (EDTA) to remove divalent cation and heavy metal impurities. Residual EDTA was removed by three washes with water. Deuterium oxide (99.8%) was obtained from Bio-Rad Laboratories (Richmond, CA) and used without further treatment. Sodium chloride was obtained from Ventron (Danvers, MA) as the Ultrapure grade. All other inorganic chemicals were reagent grade. 2-(*N*-Morpholino)ethanesulfonic acid (Mes) was obtained from P-L Biochemicals, Inc. (Milwaukee, WI). Tris-(hydroxymethyl)aminomethane (Tris) was obtained from Sigma Chemical Co. (St. Louis, MO), and 3-(*N*-morpholino)propanesulfonic acid (Mops) was obtained from Calbiochem (La Jolla, CA). H_2O was purified by passage through a Milli-Q ion-exchange system (Millipore, Bedford, MA) and had a conductivity of less than $10^{-7} \Omega^{-1}/\text{cm}^2$.

^{31}P and ^{13}C NMR Measurements. ^{31}P and ^{13}C NMR spectra of sonicated PS vesicles were obtained at 145.7 and 90.5 MHz, respectively, on a Bruker WH-360 spectrometer. The solution used for the temperature-dependence studies (see Appendix) contained 15% $^2\text{H}_2\text{O}$, and the deuterium resonance was used as a "lock". The effect of cobalt on the transverse relaxation rate, $1/T_{2P}$, was calculated from the relationship $1/T_{2P} = \pi\Delta\nu_P$, where $\Delta\nu_P$ is the increase in the line width of the NMR signal on the addition of cobalt. Broad band proton decoupling was used for the ^{13}C NMR experiments but not for the ^{31}P NMR experiments.

Sonicated PS vesicles were prepared in 0.1 M sodium chloride and 1 mM Mes, pH 5.0; the method of preparation is described elsewhere (McLaughlin et al., 1981). Each data point was determined from a fresh sample of sonicated PS vesicles, and the final phospholipid concentration was determined by phosphate analysis (Lowry & Tinsley, 1974).

For most of the experiments cobalt was added after sonication. If cobalt is added to the sample before sonication, the ^{31}P NMR signal from the inner PS molecules is approximately 90% broader than the signal from the outer PS molecules. This effect is also observed with phosphatidylcholine vesicles, where it is due to an increased binding of cobalt to the inner phosphodiester groups relative to the outer phosphodiester groups (A. C. McLaughlin, unpublished experiments). The line width of the broad ^{31}P NMR signal from outer PS molecules was calculated by using the $(\pi, t, \pi/2)$ radio-frequency pulse sequence, with a value of t that nulled the narrow ^{31}P NMR signal from the inner PS molecules (Lau et al., 1981).

A calibration curve of the ^{31}P NMR line width as a function of the free cobalt concentration was determined by using sonicated vesicles that had been passed through a Sephadex G-200 column equilibrated with a known concentration of cobalt in 0.1 M sodium chloride and 1 mM Mes, pH 5.0. In

all the other experiments, small aliquots of a concentrated cobalt solution were added directly to the sonicated vesicle suspension.

For the temperature-dependence experiments (i.e., see Figure 3) cobalt was added before sonication; if cobalt is added after sonication it diffuses into the interior of the vesicles at the high temperatures (i.e., $>40^\circ\text{C}$). For these experiments, the line width of the signal from the outer PS molecules was calculated by mathematical deconvolution of the observed signal (Lau et al., 1981).

Assignment of ^{13}C NMR Signals from the Polar Head-Group Region of PS Membranes. The two signals at approximately 174 ppm (see Figure 2) occur at the same position as the signals from the carbonyl groups of phosphatidylcholine (Sears, 1975) and phosphatidylglycerol membranes (Lau et al., 1981). The separation between the signals (0.43 ppm) is similar to the separation between the signals from the carbonyl groups on the inner and outer monolayers of sonicated dipalmitoylphosphatidylcholine vesicles (Schmidt et al., 1977). These signals are assigned to carbonyl groups on the inner (174.06 ppm) and the outer (173.63 ppm) monolayers of PS vesicles. The signal at 171.59 ppm occurs at the same position as the signal from the carboxyl groups of serine, phosphoserine, and glycerophosphorylserine (A. McLaughlin, unpublished results) and is assigned to the carboxyl group of PS.

The signals from the remaining carbon atoms in the polar head group occur between 55 and 75 ppm (see Figure 1). The signal at 70.75 ppm occurs at the same position as the signal from the glycerol C2 carbon in phosphatidylcholine (Sears, 1975) and in phosphatidylglycerol (Lau et al., 1981) vesicles and is assigned to the glycerol C2 carbon of PS. The positions of the partially resolved signals at 64.33 and 64.00 ppm are similar to the positions of the signal from the C_β methylene group in glycerophosphorylserine (64.57 ppm) and the signal from the glycerol C3 carbon (63.85) in phosphatidylglycerol membranes (Lau et al., 1981).¹ These signals are assigned to the methylene groups adjacent to the phosphodiester group in PS. The signal at 63.13 ppm occurs at the same position as the glycerol C1 carbon in phosphatidylcholine (A. McLaughlin, unpublished results) and phosphatidylglycerol membranes (Lau et al., 1981) and is assigned to the glycerol C1 carbon of PS. The signal at 55.20 ppm occurs at the same position as the C_α carbon of serine, serine phosphate, and glycerophosphorylserine (A. McLaughlin, unpublished results) and is assigned to the C_α carbon of the serine moiety in PS.

The assignment of the unsaturated and saturated carbon atoms in the fatty acid chains is similar to the assignment of these carbons in phosphatidylcholine (Sears, 1975; Metcalfe et al., 1971; Batchelor et al., 1972).

Proton Release Measurements. Sonicated PS vesicles (approximately 2 mM) were suspended in a solution containing 0.1 M sodium chloride and 10 μM of the following three buffers: Tris, Mops, and Mes. The exact PS concentration was determined by phosphate analysis. The temperature was maintained at 25°C , and argon gas was blown over the solution to prevent pH drift due to absorption of carbon dioxide.

The vesicle sample was titrated to the appropriate pH (5.0 or 7.0), and a small aliquot of cobalt was added to bring the cobalt concentration to 91 μM . The change in pH was noted and the number of protons released calculated from a standard titration curve. The concentration of bound cobalt ions was determined from the Stern equation (McLaughlin et al., 1981)

¹ In Lau et al. (1981) the assignment of the glycerol C3 carbon and of the choline CH_2OP carbon was reversed.

Table I: ^{31}P NMR Determination of Cobalt Binding to the Phosphodiester Group of PS^a

| $[\text{Co}^{2+}]_{\text{tot}} (\mu\text{M})$ | $1/T_{2\text{P}} (\text{ms}^{-1})$ | $f (\%)$ | $[\text{Co}^{2+}]_{\text{f}} (\mu\text{M})$ | $[\text{Co}^{2+}]_{\text{b}} (\mu\text{M})$ | $[\text{Co}^{2+}\text{-P}] (\mu\text{M})$ | $[\text{Co}^{2+}\text{-P}]/[\text{Co}^{2+}]_{\text{b}}$ |
|---|------------------------------------|----------|---|---|---|---|
| 131 | 0.72 | 0.085 | 1.8 | 129 | 16.7 | 0.129 |
| 298 | 1.51 | 0.178 | 3.8 | 294 | 37.0 | 0.126 |
| 458 | 2.60 | 0.307 | 6.6 | 451 | 64.5 | 0.143 |
| 648 | 3.51 | 0.414 | 8.9 | 639 | 87.0 | 0.136 |
| 763 | 4.40 | 0.519 | 11.2 | 752 | 94.1 | 0.125 |
| 996 | 5.43 | 0.640 | 13.7 | 982 | 132.2 | 0.135 |

^a The effect of cobalt on the line width of the ^{31}P NMR signal from PS molecules in the outer monolayer, $1/T_{2\text{P}}$, was determined by using the $(\pi, t, \pi/2)$ radio-frequency pulse sequence. The fraction of phosphodiester groups in the outer monolayer that are bound to cobalt in inner-sphere complexes, f , was calculated by using eq 4, assuming $\tau_{\text{M}} = 1.2 \mu\text{s}$ (see Appendix). The free cobalt concentration in the external aqueous medium, $[\text{Co}^{2+}]_{\text{f}}$, was calculated from the ^{31}P NMR line width by using a calibration curve (see Materials and Methods). The bound cobalt concentration, $[\text{Co}^{2+}]_{\text{b}}$, was calculated from the difference between the total and the free cobalt concentrations, assuming that the amount of cobalt in the double layer was negligible (Lau et al., 1981). The concentration of phosphodiester groups bound in inner-sphere complexes with cobalt, $[\text{Co}^{2+}\text{-P}]$, was calculated by using eq 5. The total PS concentration was $30.3 \pm 1.7 \text{ mM}$.

by using the association constant for the 1:1 cobalt:PS complex calculated from the ζ potential measurements ($K_2 = 28 \text{ M}^{-1}$; see Results).

Electrophoretic Mobility Measurements. The electrophoretic mobility measurements were carried out at 25°C in the presence of 0.1 M sodium chloride. The ζ potential was calculated from the measured values of the electrophoretic mobility by using the Helmholtz-Smoluchowsky equation (McLaughlin et al., 1981).

Results

^{31}P NMR Measurements. The effects of cobalt on the line width of the ^{31}P NMR signal from sonicated PS vesicles were used to determine the apparent and intrinsic association constants for the interaction of cobalt with PS. Assuming a 1:1 stoichiometry, the apparent association constant K_{A} is defined as

$$K_{\text{A}} = [\text{Co}^{2+}]_{\text{b}} / ([\text{Co}^{2+}]_{\text{f}} [\text{PS}]) \quad (1)$$

where $[\text{Co}^{2+}]_{\text{f}}$ is the free cobalt concentration, $[\text{Co}^{2+}]_{\text{b}}$ is the bound cobalt concentration, and $[\text{PS}]$ is the concentration of PS molecules available to bind cobalt (Lau et al., 1981). $[\text{Co}^{2+}]_{\text{f}}$ was calculated from the ^{31}P NMR line width by using a calibration curve (see Materials and Methods), and $[\text{Co}^{2+}]_{\text{b}}$ was calculated from the difference between the total and the free cobalt concentrations (see Table I). $[\text{PS}]$ was calculated from the total PS concentration, $[\text{PS}]_{\text{tot}}$, by using the expression (McLaughlin et al., 1981)

$$[\text{PS}] = \frac{\alpha [\text{PS}]_{\text{tot}}}{1 + K_1 C^+ \exp[-F\psi_0/(RT)]} \quad (2)$$

where α , the fraction of PS molecules in the outer monolayer, was calculated to be 0.67 at pH 5.0, from manganese titrations of the observed ^{31}P NMR signal (A. McLaughlin, unpublished experiments). The denominator in eq 2 accounts for the postulated direct competition between sodium and cobalt for the same binding sites. K_1 is the intrinsic sodium-PS association constant, ψ_0 is the surface potential, C^+ is the sodium concentration in the bulk aqueous medium, and F is Faraday's constant. The reported values of K_1 range from 0.6 to 1 M^{-1} (Nir et al., 1978; Eisenberg et al., 1979; Kurland et al., 1979b; Ohki & Kurland, 1981). When these values of K_1 are used, the calculated values of ψ_0 at pH 5.0 range from -83 to -76 mV (Eisenberg et al., 1979), and the calculated values of K_{A} range from 8.7×10^3 to $10 \times 10^3 \text{ M}^{-1}$.

Assuming a 1:1 stoichiometry, the intrinsic association constant, K_2 , is related to K_{A} by the equation

$$K_2 = K_{\text{A}} \exp[2F\psi_0/(RT)] \quad (3)$$

By use of the values of K_{A} and ψ_0 given above, the calculated

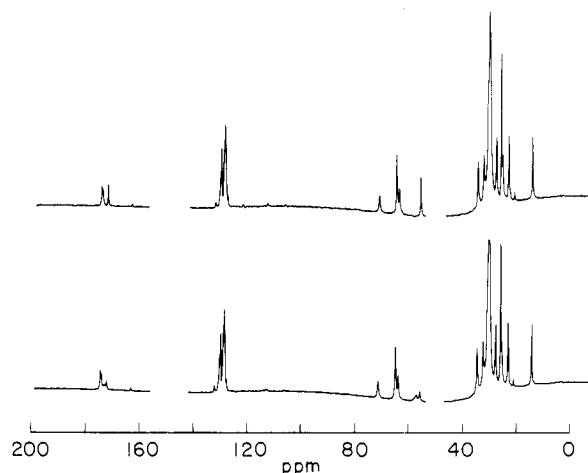


FIGURE 1: ^{13}C NMR spectrum of sonicated PS vesicles in the absence (top) and presence (bottom) of $303 \mu\text{M}$ cobalt (total) in the bulk aqueous medium. The signals are assigned in the text (see Materials and Methods). The ppm scale is downfield from tetramethylsilane. $T = 20^\circ\text{C}$. The total PS concentration was 108 mM ; other conditions are given in the text.

values of K_2 are between 14 and 27 M^{-1} . These values can be compared with the value of K_2 calculated from the electrophoresis experiments, 28 M^{-1} (see below). The agreement is considered satisfactory.

At 20°C the line width of the ^{31}P NMR signal, $1/T_{2\text{P}}$, is given by the expression

$$1/T_{2\text{P}} = f/\tau_{\text{M}} \quad (4)$$

where f is the fraction of phosphodiester groups bound in inner-sphere complexes with cobalt and τ_{M} is the lifetime of the complex (Swift & Connick, 1962). The observed values of $1/T_{2\text{P}}$ at 20°C were used to calculate f , assuming $\tau_{\text{M}} = 1.2 \mu\text{s}$ at this temperature (see Appendix). The concentration of phosphodiester groups bound in inner-sphere complexes, $[\text{Co}^{2+}\text{-P}]$, was calculated from the expression

$$[\text{Co}^{2+}\text{-P}] = f\alpha [\text{PS}]_{\text{tot}} \quad (5)$$

The calculated value of $[\text{Co}^{2+}\text{-P}]$ is approximately 13% of the value of $[\text{Co}^{2+}]_{\text{b}}$ for all the samples (see Table I). If the stoichiometry of the inner-sphere complex of cobalt with the phosphodiester group of PS is 1:1, this result implies that only 13% of the bound cobalt ions are involved in inner-sphere complexes with the phosphodiester group.

^{13}C NMR Measurements. The complete ^{13}C NMR spectrum of sonicated PS vesicles is shown in Figure 1. The signals between 15 and 35 ppm and the signals at approximately 130 ppm arise from saturated carbons and unsaturated carbons, respectively, in the fatty acid chains (Metcalf et al., 1971;

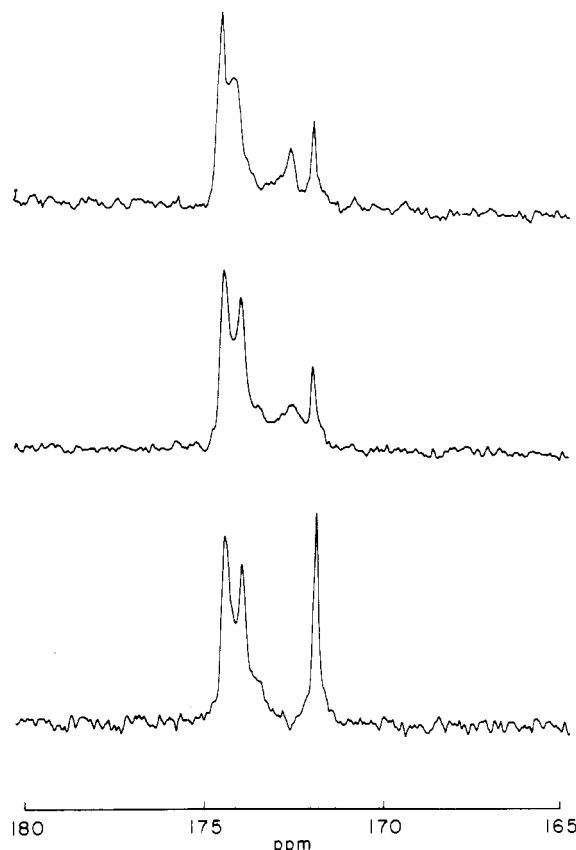


FIGURE 2: Carboxyl (~ 172 ppm) and carbonyl (~ 174 ppm) region of the ^{13}C NMR spectrum of sonicated PS vesicles in the absence and presence of cobalt. (Bottom) No cobalt, $T = 20^\circ\text{C}$; (middle) $303\ \mu\text{M}$ cobalt, $T = 20^\circ\text{C}$; (top) $303\ \mu\text{M}$ cobalt, $T = 40^\circ\text{C}$. The ppm scale is downfield from tetramethylsilane. The total PS concentration was $108\ \text{mM}$; other conditions are given in the text.

Batchelor et al., 1972). The signals between 165 and 170 ppm arise from the carbonyl and carboxyl carbons and are shown in more detail in Figure 2. The signals between 50 and 75 ppm arise from the remaining carbons in the polar head group. The assignments of the signals from carbons in the polar head-group region is discussed in detail under Materials and Methods.

Upon the addition of cobalt ($303\ \mu\text{M}$) to sonicated PS vesicles, the intensity of the ^{13}C NMR signal from the carboxyl group ($171.59\ \text{ppm}$) decreased to approximately one-third of its original value, and a new signal appeared $0.619\ \text{ppm}$ downfield from the original signal (see Figure 2). The unshifted signal is assigned to carboxyl groups of PS molecules on the inner surface of the vesicles, and the shifted signal is assigned to carboxyl groups of PS molecules on the outer surface of the vesicles.

Model studies with serine suggest that cobalt forms two types of complexes with the carboxyl group of PS—a unidentate complex involving only the carboxyl group and an intramolecular chelate complex involving both the carboxyl group and the amine group (Freeman, 1973). These two complexes can be distinguished by their different ^{13}C NMR signals. The ^{13}C NMR signal from the carboxyl group in the unidentate complex between cobalt and serine is shifted $25\ \text{kHz}$ downfield, and the lifetime of this complex is $0.8\ \mu\text{s}$ at 40°C ; the ^{13}C NMR signal from the carboxyl group in the chelate complex between cobalt and serine is shifted $17\ \text{kHz}$ upfield, and the lifetime of the carboxyl group in this complex is $\sim 2\ \text{ms}$ at 25°C (A. C. McLaughlin, unpublished experiments). If the lifetime of the chelate complex between cobalt

and the carboxyl group of PS is similar to the lifetime of the chelate complex between cobalt and the carboxyl group of serine, the effects of this complex will not be exchange narrowed;² the ^{13}C NMR signal from carboxyl groups in the chelate complex will be shifted approximately $17\ \text{kHz}$ upfield but will be too broad to observe. If the lifetime of the unidentate complex between cobalt and the carboxyl group of PS is similar to the lifetime of the unidentate complex between cobalt and the carboxyl group of serine, the effects of this complex will be exchange narrowed; the position of the observed ^{13}C NMR signal from the carboxyl group will be the weighted average of the positions of the signals from free carboxyl groups and carboxyl groups bound in unidentate complexes.

When the temperature was raised from 20 to 40°C , the width of the downfield-shifted ^{13}C NMR signal from the carboxyl groups of PS decreased to approximately $30\ \text{Hz}$, but the shift, $\Delta\omega_p$, increased by less than 10% (see Figure 2); these results confirm that the observed effects of cobalt on this signal are exchange narrowed. Under these conditions $\Delta\omega_p$ is given by

$$\Delta\omega_p = f\Delta\omega_M \quad (6)$$

where $\Delta\omega_M$ is the shift of the ^{13}C NMR signal from the carboxyl group in the unidentate inner-sphere complex and f is the fraction of carboxyl groups involved in this complex (Swift & Connick, 1962). By use of eq 6, f is calculated to be 2.2×10^{-3} at 20°C , assuming $\Delta\omega_M$ is $25\ \text{kHz}$. When this value of f was used, the concentration of carboxyl groups bound in unidentate inner-sphere complexes, $[\text{Co}^{2+}\text{--CO}_2]$, was calculated to be $161\ \mu\text{M}$ from the expression

$$[\text{Co}^{2+}\text{--CO}_2] = f\alpha[\text{PS}]_{\text{tot}} \quad (7)$$

assuming that $\alpha = 0.67$ (see above).

$[\text{Co}^{2+}\text{--CO}_2]$ can be compared with the bound cobalt concentration, $[\text{Co}^{2+}]_b$, determined from the difference between the total cobalt concentration and the free cobalt concentration (calculated by using the data shown in Table I). $[\text{Co}^{2+}\text{--CO}_2]$ ($161\ \mu\text{M}$) is approximately 54% of $[\text{Co}^{2+}]_b$ ($301\ \mu\text{M}$). If the stoichiometry of the inner-sphere complex is $1:1$, this result implies that only 54% of the bound cobalt ions are involved in unidentate inner-sphere complexes with the carboxyl group.

Under exchange-narrowed conditions the width, $1/T_{2p}$, of the downfield-shifted signal from the carboxyl group is given by the equation³ (Swift & Connick, 1962)

$$1/T_{2p} = f\Delta\omega_M^2\tau_M \quad (8)$$

By use of eq 8, τ_M is estimated to be approximately $1.6\ \mu\text{s}$ at 40°C .

The addition of cobalt ($303\ \mu\text{M}$) also decreased the intensity of the signal from the C_α carbon of the serine moiety ($55.2\ \text{ppm}$) and generated a new signal $1.48\ \text{ppm}$ downfield. The unshifted signal is assigned to the C_α carbon of PS molecules on the inner monolayer, while the shifted signal is assigned to the C_α carbon of PS molecules on the outer monolayer. Although the shift of the C_α carbon is approximately twice the shift of the carboxyl carbon, two lines of evidence suggest that it arises from the inner-sphere complex of cobalt with the carboxyl group, rather than from an inner-sphere complex with

² Exchange narrowing is defined by the condition $\Delta\omega_M\tau_M \ll 1$ (McLaughlin & Leigh, 1973).

³ Equation 7 assumes the condition $1/T_{2M} \ll \Delta\omega_M^2\tau_M$. This condition has been verified for the effects of cobalt on the ^{31}P NMR signal of inner sphere phosphodiester groups (McLaughlin et al., 1978a). Preliminary experiments with serine suggest it is also valid for the effects of cobalt on the ^{13}C NMR signal from unidentate inner sphere carboxyl groups (A. C. McLaughlin, unpublished experiments).

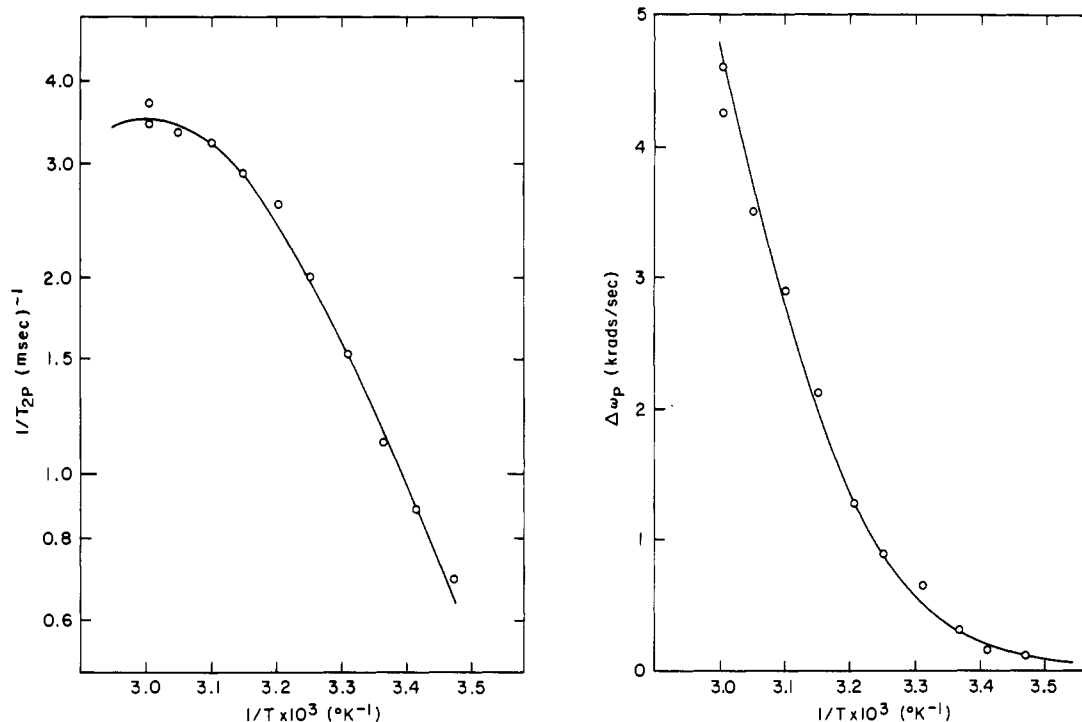


FIGURE 3: Effect of cobalt on the line width, $1/T_{2p}$, and the position, Δw_p , of the ^{31}P NMR signal from the outer phosphodiester groups in sonicated phosphatidylserine vesicles. The solid lines were calculated from the simultaneous best fit of the data to eq 9 and 10 (see Appendix). The total cobalt concentration was 373 μM , and the total PS concentration was 30 mM. Other conditions are given in the text.

the amine group or the phosphodiester group. First, similar shifts are seen in cobalt complexes with serine at low pH values, where the concentration of the chelate complex is negligible (A. McLaughlin, unpublished results). Second, when a divalent cation binds to oxygen or nitrogen ligands, hyperfine effects on the ^{13}C NMR signals of the neighboring carbon atoms are usually larger for the second carbon than for the carbon atom immediately adjacent to the ligand. For example, the interaction of nickel with the amine group of *n*-propylamine produces a larger shift of the C_β carbon (-460 ppm) than of the C_α carbon ($+120$ ppm) (Strouse & Matwiyoff, 1970).

Cobalt did not affect the shift of the carbonyl signals (174.06 and 173.63 ppm; see Figure 3), indicating that the carbonyl groups do not form inner-sphere complexes with cobalt. As expected, the signals from the other carbons in the polar head group, i.e., the glycerol C1, C2, and C3 and the serine C_β , were not affected by the low cobalt concentrations used in these experiments.

Potential Measurements. The effect of cobalt on the ζ potential of PS membranes has been investigated at pH 7.0 (McLaughlin et al., 1981). It was concluded that the data at this pH can be explained by the Gouy-Chapman-Stern theory with an apparent association constant, K_2 , of 28 M^{-1} . These studies have been extended to lower pH values. In the absence of cobalt, the ζ potential changed by less than 10% when the bulk pH was lowered from 7.0 to 5.0, in agreement with previous results (Papahadjopoulos, 1968; Hauser et al., 1976). This small change in the ζ potential indicates that the carboxyl group is not substantially protonated at pH 5.0, which is consistent with calculations that suggest approximately 90% of the carboxyl groups are ionized at this pH.⁴

⁴ The apparent pK , i.e., the pH at which 50% of the carboxyl groups are ionized, is assumed to be 3.5 (MacDonald et al., 1976). The degree of ionization does not follow a simple Henderson-Hasselbalch formulation because the surface potential changes during the titration of the carboxyl group (MacDonald et al., 1976; McLaughlin, 1977).

The effect of cobalt (10^{-4} – 10^{-1} M) on the ζ potential of PS membranes was identical, to within experimental error, at pH 5.0, 6.0, and 7.0 (data not shown).

Proton Release Studies. Both the carboxyl group and the amine group form inner-sphere ligands for cobalt in the chelate complex, and the formation of the chelate complex at pH values substantially lower than the pK of the amine group (~ 9.2) results in the release of a proton from this group (Albert, 1950). Proton release studies with sonicated PS vesicles showed that 1.05 ± 0.17 (SD, $N = 9$) protons were released for every cobalt ion bound to the PS membranes at pH 7.0, while 0.32 ± 0.02 (SD, $N = 5$) proton was released for every bound cobalt ion at pH 5.0.

Cobalt complexes that involve chelation between the carboxyl group and the amine group are not considered realistic models for complexes between alkaline earth cations and PS (see Discussion). For minimization of the concentration of these complexes, the pH must be low enough so that protons can successfully compete with cobalt for the amine group. While a bulk pH value lower than 5.0 would decrease the residual binding to the amine group, it would also increase the protonation of the carboxyl group (see above). The pH value of 5.0 was chosen as a compromise between these two effects.

Discussion

PS contains four types of functional groups that could bind divalent cations: the phosphodiester group, the carboxyl group, the amine group, and the carbonyl group. The results presented here identify the groups that form inner-sphere complexes with cobalt. The ^{31}P NMR results show that 13% of the bound cobalt ions are involved in inner-sphere complexes with the phosphodiester group, and the ^{13}C NMR results show that 54% of the bound cobalt ions are involved in unidentate inner-sphere complexes with the carboxyl group. No evidence is found for cobalt binding to the carbonyl groups, but the proton release studies suggest that approximately 32% of the bound cobalt ions are involved in chelate complexes with the

carboxyl and the amine groups.⁵

The calculation of the number of cobalt ions involved in unidentate inner-sphere complexes with the carboxyl group of PS does not include cobalt ions involved in chelate complexes containing both the carboxyl and the amine groups (see Results). Most (i.e., 13% + 54% + 32% = 99%) of the cobalt ions bound to the surface of PS membranes can thus be accounted for by inner-sphere complexes with the phosphodiester group or the carboxyl group, and only a small fraction of the bound cobalt ions appear to be involved in outer-sphere complexes, i.e., weak "ion pairs" formed between the charged ligand and the completely hydrated divalent cation (Basolo & Pearson, 1967; Hewkin & Prince, 1970).⁶ The distribution of bound cobalt ions between inner- and outer-sphere complexes in PS is substantially different from the distribution found for phosphatidylglycerol, where approximately 80% of the bound cobalt ions are involved in outer-sphere complexes (Lau et al., 1981). The difference between the two types of phospholipids is the carboxyl group of PS, which is the principle binding site for cobalt ions in these membranes.

The NMR results cannot be used to directly distinguish unidentate inner-sphere complexes involving only the carboxyl group from intramolecular chelate complexes involving inner-sphere ligands to both the carboxyl and the phosphodiester groups. The results suggest, however, that this type of chelate complex is relatively unimportant, because only 13% of the bound cobalt ions are involved in inner-sphere complexes with the phosphodiester group. This suggestion is consistent with the concept that five- or six-membered intermolecular chelate complexes are stable, but larger rings generally are not (Sigel & McCormick, 1970; Freeman, 1973). The intramolecular chelate complex involving the carboxyl group and the amine group forms a five-membered ring, while an intramolecular chelate complex involving the carboxyl group and the phosphodiester group would form an eight-membered ring.

Complexes between cobalt and PS are important because they provide precise molecular information about the binding sites for divalent cations. Structural features of the complexes between cobalt and PS that are relevant to the complexes between alkaline earth cations and PS can be determined from model studies. For example, the lack of discrimination between divalent transition metal and alkaline earth cations in complexes with phosphate monoester ligands and carboxylate ligands (Sigel & McCormick, 1970) suggests that the inner-sphere complex between cobalt and the phosphodiester group of PS and the unidentate inner-sphere complex between cobalt and the carboxyl group of PS are reasonable models for complexes between alkaline earth cations and PS. This is not true, however, for amine ligands; the association constant for formation of the chelate complex with glycine is approximately 4 orders of magnitude larger for cobalt than for calcium (Sigel & McCormick, 1970), which suggests that the chelate complex of cobalt with the carboxyl group and the amine group of PS is not a reasonable model for complexes between alkaline earth ions and PS.

The above conclusions are consistent with the results of Hendrickson & Fullington (1965) on the effects of nickel and calcium on the pH titration curves of PS. At low pH values, where nickel does not form chelate complexes, the apparent association constants for nickel and calcium are very similar. However, at high pH values, where nickel does form chelate

complexes, the apparent association constant for nickel increases by approximately 4 orders of magnitude, while the apparent association constant for calcium is independent of pH. Similar results would be expected for the cobalt complexes (Sharma, 1967).

A variety of models for the complex between calcium and PS have been proposed. Hauser et al. (1969) suggested that calcium binds only to the phosphodiester group of PS. On the other hand, Kurland et al. (1979a,b) suggested that calcium binds to PS in a bidentate complex that involves both the phosphodiester group and the carboxyl group, and Papahadjopoulos (1968) suggested that calcium binds in a chelate complex that involves ligands to the phosphodiester, carboxyl, and amine groups. Our studies with cobalt suggest that calcium probably forms inner-sphere complexes with both the phosphodiester group and the carboxyl group and suggest that the carboxyl group is the preferred binding site. The results with cobalt also suggest that calcium does not form intramolecular chelate complexes involving the carboxyl group and the phosphodiester group or intramolecular chelate complexes involving the carboxyl group and the amine group.

It should be emphasized that, under the experimental conditions employed in these studies, only a small fraction (i.e., <1%) of PS molecules is bound to cobalt. The complexes formed under these conditions may be different from those formed in solutions of higher divalent cation concentrations (Hauser et al., 1977; Hope & Cullis, 1980) where a large fraction (~40%) of PS molecules are bound to calcium and where extensive aggregation and morphological changes occur (Papahadjopoulos et al., 1975; van Dijck et al., 1978; Portis et al., 1979).

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Appendix

Calculation of the Lifetime of the Cobalt-Phosphodiester Complex in PS Membranes. The determination of the lifetime of the cobalt-phosphodiester complex, τ_M , has been discussed in detail previously (McLaughlin et al., 1978a,b). Briefly, it involves fitting the temperature dependence of the effect of cobalt on the width, $1/T_{2P}$, and shift, $\Delta\omega_P$, of the ^{31}P NMR signal to the following equations:

$$1/T_{2P} = f \left(\frac{\Delta\omega_M^2 \tau_M}{1 + \Delta\omega_M^2 \tau_M^2} \right) \quad (9)$$

$$\Delta\omega_P = f \left(\frac{\Delta\omega_M}{1 + \Delta\omega_M^2 \tau_M^2} \right) \quad (10)$$

where f is the fraction of phosphate groups involved in inner-sphere complexes with cobalt and $\Delta\omega_M$ is the shift of the ^{31}P NMR signal for the complex. $\Delta\omega_M$ cannot be determined directly for the membrane systems but can be estimated from studies on soluble fragments of phospholipid molecules (McLaughlin et al., 1978a). We used the value of $\Delta\omega_M$ determined for glycerylphosphorylglycerol (McLaughlin, 1982), because the most appropriate model compound for PS, glycerophosphorylserine, is unstable under the conditions necessary for these experiments. The temperature dependence of $\Delta\omega_M$ and f is assumed to be of the form $\tau_M = \tau_M^0 \exp[\Delta H_1/(RT)]$ and $f = f^0 \exp[\Delta H_2/(RT)]$, and τ_M is assumed to be inversely proportional to temperature (Bloembergen,

⁵ This calculation ignores higher order (i.e., 2:1 or 3:1) complexes (Sharma, 1967).

⁶ The accuracy of this calculation is estimated to be approximately $\pm 20\%$.

1957). ΔH_1 was calculated to be 8.2 kcal/mol from the temperature dependence of the ratio of $1/T_{2P}$ to $\Delta\omega_p$, as previously described (McLaughlin, 1982). By use of this value of ΔH_1 , the values of ΔH_2 (-3.2 kcal/mol), f^0 (0.26), and τ_M^0 (0.90×10^{-12}) were calculated from the simultaneous best fit of $1/T_{2P}$ and $\Delta\omega_p$ to eq 9 and 10 (see Figure 3). τ_M is calculated to be 1.2 μ s at 20 °C, using the values of ΔH_1 and τ_M^0 given above.

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