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THE ADSORPTION OF DIVALENT CATIONS TO PHOSPHATIDYLGLYCEROL BILAYER MEMBRANES

ARTHUR LAU, ALAN McLAUGHLIN [a,*] and STUART McLAUGHLIN [b]

[a] *Biology Department, Brookhaven National Laboratory, Upton, NY 11973 and* [b] *Department of Physiology and Biophysics, Health Sciences Center, SUNY, Stony Brook, Long Island, NY 11794 (U.S.A.)*

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The ability of the Stern equation to describe the adsorption of divalent cations to phosphatidylglycerol membranes was tested by combining ^{31}P-NMR and electrophoretic mobility measurements. In 0.1 M sodium chloride both the ^{31}P-NMR and the zeta potential data are well described by the Stern equation. ^{31}P-NMR and ^{13}C-NMR results indicate that cobalt forms inner-sphere complexes only with the phosphate group of phosphatidylglycerol molecules and that a substantial fraction of the adsorbed cobalt ions form outer-sphere complexes. Evidence is presented that suggests the alkaline earth cations also bind to phospholipids mainly by forming outer sphere complexes. Electrophoretic mobility measurements were performed with several different divalent cations. In all cases the zeta potentials in 0.1 M sodium chloride were well described by the Stern equation. The intrinsic 1 : 1 association constants (M^{-1}) for the phosphatidylglycerol complexes decreased in the sequence: Mn^{2+}, 11.5; Ca^{2+}, 8.5; Ni^{2+}, 7.5; Co^{2+}, 6.5; Mg^{2+}, 6.0; Ba^{2+}, 5.5 and Sr^{2+}, 5.0.

Introduction

Bilayer membranes formed from a mixture of zwitterionic and negative phospholipids provide a convenient model system for an investigator interested in the interaction of divalent cations with biological membranes. However, a number of theoretical and experimental problems arise even with this simple system. One problem is that the binding of divalent cations to membranes cannot be measured directly with most experimental techniques. Equilibrium dialysis [1] and ion-sensitive electrode [2] experiments, for example, measure the loss of divalent cations from the aqueous medium, but do not provide information about the structure of the bound phospholipid complexes. They cannot be used to distinguish between inner-sphere complexes, where the ligand is inserted into the first coordination sphere of the divalent cation, and outer-sphere complexes, where the charged ligand and the fully-hydrated divalent cation form an 'ion-pair' [3,4]. They also cannot be used to determine the binding of divalent cations to individual lipids in membranes formed from a mixture of phospholipids.

These problems may be circumvented by studying the effects of paramagnetic divalent transition metal cations on the nuclear magnetic resonance (NMR) spectra of phospholipid molecules. In sonicated vesicles phospholipid molecules show high-resolution NMR spectra [5–7], and the effects of paramagnetic divalent cations on the spectra can be calibrated to determine directly the number of these divalent cations bound in inner-sphere complexes [8–11]. NMR signals from the different components of membranes formed from mixtures of phospholipids can be resolved [12,13], and the number of paramagnetic divalent cations involved in inner-sphere complexes with each component determined. Because the effects of cobalt on model compounds are easiest to quanti-

* To whom correspondence should be addressed.
Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; Mops, 4-morpholinepropanesulphonic acid.

tate [8,9], it is the most useful paramagnetic divalent transition metal cation for this purpose.

The binding of divalent cations depends on the surface charge density of the membrane. In terms of the Gouy-Chapman-Stern [14] model the effects of surface charge may be theoretically described by the combination of three equations: the Grahame [15,16], Boltzmann and Langmuir [16] relations. Although the Gouy-Chapman-Stern theory has been used to interpret the interaction of divalent cations with biological membranes (e.g Ref. 17), the theory contains a number of questionable assumptions and its validity must be experimentally tested in model systems.

We previously tested the ability of the Gouy-Chapman-Stern theory to describe the adsorption of divalent cations to neutral bilayer membranes by using ^{31}P-NMR to estimate the number of bound cobalt ions and zeta potential measurements to estimate the surface potential [11]. We concluded that the theory could adquately describe the binding of divalent cations to neutral membranes formed from phosphatidylcholine. The results reported here represent an extension of this work to negatively charged bilayer membranes formed from phosphatidylglycerol. Although phosphatidylglycerol, the major negative lipid in bacterial [18] and plant [19] membranes, is generally supplanted by phosphatidylserine in animal membranes [20] it has two advantages for these studies. First, the structure of the polar head group region of phosphatidylglycerol is simpler than that of phosphatidylserine. Second, the phase behavior of membranes formed from mixtures of phosphatidylglycerol and phosphatidylcholine is simpler than the phase behavior of membranes formed from mixtures of phosphatidylserine and phosphatidylcholine [21–23]. It should therefore be easier to extend the experiments presented here to membranes formed from mixture of phosphatidylglycerol and phosphatidylcholine.

Theory

The Stern equation is a combination of the Grahame equation, the Boltzmann relation and the Langmuir adsorption isotherm. The Grahame equation from the theory of the diffuse double layer relates the surface charge density, σ, and the surface potential, ψ_o:

$$\sigma^2 = 2\epsilon_r\epsilon_o RT \sum_i C_i[\exp(-z_i F\psi_o/RT) - 1] \quad (1)$$

where C_i is the concentration of ions of valence z_i in the bulk aqueous phase, ϵ_r is the dielectric constant of the aqueous phase, ϵ_o is the permittivity of free space, R is the gas constant, T the temperature, and F the Faraday constant [14–16]. The Boltzmann equation relates the concentrations of monovalent and divalent cations in the aqueous phase at the surface of the membrane, $C^+(0)$ and $C^{2+}(0)$, to the bulk concentrations, C^+ and C^{2+}, and the surface potential:

$$C^+(0) = C^+\exp(-F\psi_o/RT)$$
$$C^{2+}(0) = C^{2+}\exp(-2F\psi_o/RT) \quad (2)$$

The Langmuir adsorption isotherm relates the surface concentration of 1 : 1 complexes formed between phosphatidylglycerol and the monovalent cation, $\{C^+P^-\}$, to the free surface concentration of the negative lipid, $\{P^-\}$, and the local concentration of the monovalent cation, $C^+(0)$:

$$\{C^+P^-\} = K_1\{P^-\}C^+(0) \quad (3)$$

where K_1 is an intrinsic association constant and the braces denote a surface concentration. If we assume that divalent cations form only 1 : 1 complexes with phosphatidylglycerol, the Langmuir adosprtion isotherm predicte that:

$$\{C^{2+}P^-\} = K_2\{P^-\}C^{2+}(0) \quad (4)$$

where K_2 is an intrinsic association constant and $\{C^{2+}P^-\}$ is the surface concentration of 1 : 1 complexes formed between the negative lipid and the divalent cations. The total surface concentration of the negative lipid, $\{P^-\}^{tot}$, is the sum of the free and the bound concentrations:

$$\{P^-\}^{tot} = \{P^-\} + \{C^+P^-\} + \{C^{2+}P^-\} \quad (5)$$

Algebraic manipulation of Eqns. 3–5 yields:

$$\sigma = \frac{-\{P^-\}^{tot}(1 - K_2C^{2+}(0))}{(1 + K_1C^+(0) + K_2C^{2+}(0))} \quad (6)$$

The combination of Eqns. 1, 2 and 6 is defined as the Stern equation. The Stern equation can be solved numerically for the number of bound divalent cations as a function of the concentration of divalent cations in the bulk aqueous phase. Alternatively, the Stern equation can be solved numerically for the surface potential. The theoretical value of the zeta potential, the potential at a distance of about 2 Å from the surface of the membrane [24], is calculated from ψ_o by means of diffuse double layer theory [25], assuming that phosphatidylglycerol occupies an area of 70 $Å^2$ [26].

Materials and Methods

Materials. Phosphatidylglycerol (PG) (Avanti Biochemicals, Birmingham, AL) was synthesized from egg phosphatidylcholine (egg PC) by the transphosphatidylation reaction of Yang et al. [27]. The final product was partitioned against 0.5% EDTA to remove divalent cations, and the residual EDTA was removed by three washes with water.

Deuterium oxide (99.8%) obtained from Bio-Rad Laboratories (Richmond, CA) was used without further treatment. Sodium chloride was obtained from Ventron (Danvers, MA) as the 'Ultrapure' grade. All other inorganic chemicals were reagent grade.

4-Morpholinepropanesulphonic acid (Mops) was obtained from Calbiochem, (La Jolla, CA). X-537A was a gift from Dr. W.E. Scott of Hoffman-La Roche Inc. (Nutley, NJ). H_2O was purified by passage through a Milli-Q ion exchange system (Millipore, Bedfore, MA) and has a conductivity of less than 10^{-7} mho/cm^2.

Preparation of sonicated PG vesicles. The method for preparation of sonicated vesicles is given elsewhere [2]. The final phospholipid concentrations were determined by phosphate analysis [28].

^{31}P-NMR and ^{13}C-NMR measurements. ^{31}P-NMR and ^{13}C-NMR spectra of sonicated PG vesicles were obtained at 145.7 MHz and 90.5 MHz, respectively, using a Bruker WH-360 spectrometer. The probe temperature was maintained at 20°C with a Bruker BST-100/700 unit and in some experiments the deuterium resonance of the solvent (20% ^2H_2O) was employed as a field-frequency 'lock'. $1/T_{2P}$ was calculated by using the relationship $1/T_{2P} = \pi\Delta\nu_P$, where $\Delta\nu_P$ is the difference between the ^{31}P-NMR linewidths of identical PG vesicle samples in the presence or absence of cobalt.

Determination of the ^{31}P-NMR linewidth of sonicated PG vesicles as a function of free cobalt concentration. Sonicated PG vesicles were prepared in 0.1 M (or 0.03 M) sodium chloride, 10 μM X-537A, 1 mM Mops, pH 7.5. The vesicles were dialysed against identical solutions containing cobalt chloride and then were dialysed against appropriate solutions to remove X-537A and to provide 20% ^2H_2O for the 'lock'. Control experiments showed that the free cobalt concentration in the intravesicular space and the external aqueous medium was fully equilibrated with the free cobalt concentration in the dialysis solutions and that the prolonged dialysis did not produce a significant adsorption of heavy metal contaminants to be charged vesicles. The ^{31}P-NMR spectrum of the dialysed sample was determined at 20°C. Each experimental point was determined for a fresh PG sample.

Because the association constant for cobalt to PG molecules on the inner monolayer of the vesicles is approx. 60% larger than the association constant for cobalt to PG molecules on the outer monolayer (Lau, A. and McLaughlin, A.C., unpublished results) the ^{31}P-NMR signal from the inner PG molecules is broader than that from the outer PG molecules. Thus, the observed ^{31}P-NMR spectrum is the superposition of a narrow signal from the outer PG molecules and a broad signal from the inner PG molecules. The intensity of the signal from the outer PG molecules is double the intensity of the signal from the inner PG molecules (Lau, A. and McLaughlin, A.C., unpublished results). The resulting spectrum can be mathematically deconvoluted to obtain the linewidth of the signal for the outer PG molecules. Using the deconvolution procedure, the linewidth of the signal from the outer PG molecules was found to be 85% of the full-width at half-height of the observed composite spectrum.

Determination of the ^{31}P–NMR linewidth of sonicated PG vesicles as function of total cobalt concentration. Sonicated PG vesicles were prepared in 0.1 M sodium chloride, 2 mM Mops, pH 7.5. A fresh PG sample was prepared for each experiment. Because cobalt does not bind equally to PG molecules on the inner and outer monolayers a specified amount of cobalt was added after sonication and was present

only in the external aqueous medium. The phosphorus T_1 relaxation time for PG molecules in the outer monolayer was substantially reduced by the paramagnetic effect of cobalt [8], while the phosphorus T_1 relaxation time for PG molecules in the inner monolayer was similar to the value observed in the absence of cobalt. This difference in T_1 values was used to distinguish the ^{31}P-NMR signals from the inner and outer PG molecules.

When the (π-τ-π/2) radio-frequency pulse sequence [29] is used, the intensity of the ^{31}P-NMR signals from the inner and outer PG molecules is a function of the delay time, τ. For values of τ much shorter than either T_1 value both signals are negative. Under these conditions the observed ^{31}P-NMR spectrum is similar to the usual spectrum, but inverted in sign. For values of τ much longer than either T_1 value both signals are positive and the observed ^{31}P-NMR spectrum is identical to the usual spectrum. The intensity of each signal is zero when $\tau \cong T_1$ ln2. The intensity of each signal passes through zero at very different values of τ because the T_1 values for the inner and outer PG molecules are quite different. By choosing the value of τ that nulls the signal from the inner PG molecules, the spectrum of the outer PG molecules may be determined (see Fig. 1).

The spectra shown in Fig. 1 were obtained with a series of τ values that approximately nulled the signal from the inner PG molecules. While the narrow signal from the inner PG molecules is negative for τ = 0.25 s, zero for τ = 0.34 s, and positive for τ = 0.40 2, the broad signal from the outer PG molecules is positive for these values of τ. Control experiments showed that no significant amount of cobalt diffused through the membrane during the experiments, which lasted approx. 2 h.

Determination of the ^{31}P-NMR linewidth of PG vesicles in the presence of cobalt and calcium. Because the PG vesicles eventually aggregated with the calcium concentrations used these experiments (<200 μM), the sonicated PG vesicles were passed over a Sephadex G-200 column that had been equilibrated

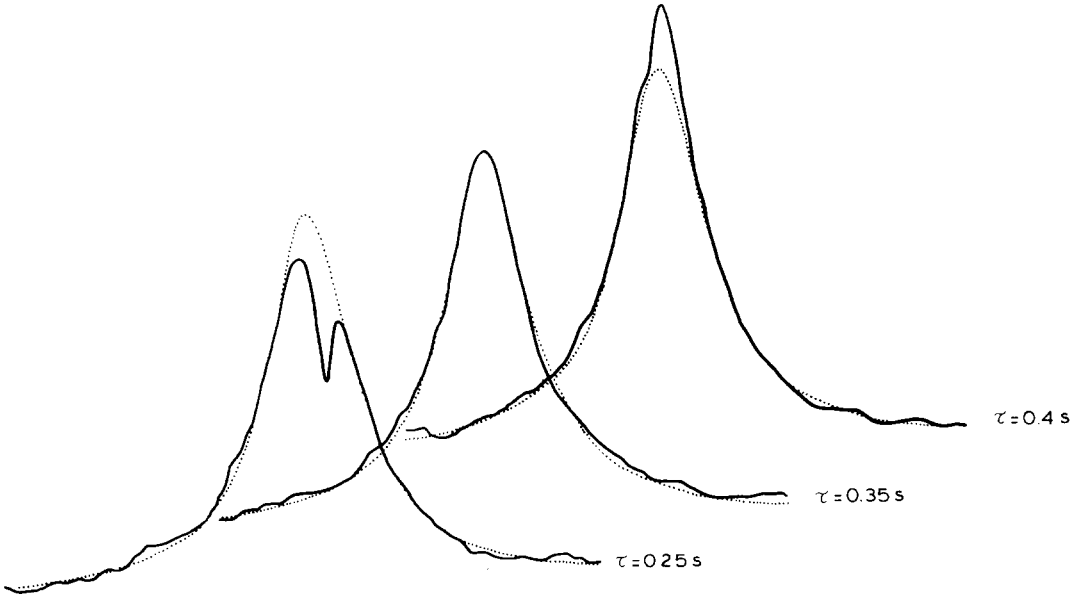

Fig. 1. ^{31}P-NMR spectra of sonicated PG vesicles. Cobalt (208 μM) was added after sonication and was present only in the external aqueous medium. The spectra were taken with the (π-τ-π/2) radio-frequency pulse sequence using the values of τ indicated in the figure. The observed spectra are the sum of two components, a broad signal from PG molecules in the outer monolayer and a narrow signal from PG molecules in the inner monolayer. The spectra were deconvoluted into the two components by assuming that the broad signal from PG molecules in the outer monolayer was Lorentzian (dotted lines). The solutions contained 0.1 M sodium chloride, 2 mM Mops, pH 7.5. The spectra were taken at 20°C and the full width of each spectrum is 4500 Hz.

with known free concentrations of cobalt and calcium in 0.1 M sodium chloride, 2 mM Mops, pH 7.5. The elution time for the vesicles was approx. 2 h, as compared to 24 h for the dialysis porcedure. A fresh PG sample was used for each experiment.

The linewidth of the ^{31}P-NMR signal for the outer PG molecules was measured as described in the preceding section. Control experiments showed that the vesicle sample was equilibrated with the free cobalt and that neither cobalt nor calcium leaked into the vesicles during the course of the experiments.

Determination of the electrophoretic mobility and calculation of the zeta potential of unsonicated PG vesicles. The multilamellar vesicles for the electrophoresis experiments were formed according to Bangham et al. [30]. Electrokinetic mobilities were measured with Rank Bros. Mark I microelectrophoresis machines (Bottisham, Cambridge, U.K.). All measurements were made at the stationary layer [31] and the current was monitored to check for electrode polarization. The zeta potential, ζ, the electrostatic potential at the hydrodynamic plane of shear, was calculated from the measured value of the electrophoretic mobility, μ, by the Helmholtz-Smoluchowski equation [32,33]: $\zeta = \mu\eta/\epsilon_r\epsilon_o$ where η is the viscosity of the aqueous phase, ϵ_r is the dielectric constant of the aqueous phase, and ϵ_o is the permittivity of free space. We assume in our analysis of the data that the hydrodynamic plane of shear is 2 Å from the surface of the membtrane and that profile of the potential in the aqueous phase may be described by the classic theory of the diffuse double layer [2,24].

Results

Zeta potential measurements

In the absence of divalent cations the observed zeta potential of multilamellar PG vesicles in 0.1 M sodium chloride is approx. −60 mV (see Fig. 2). This value is consistent with the zeta potential calculated from the Gouy-Chapman-Stern theory (Eqns. 1, 2 and 6), assuming that the plane of shear is 2 Å from the surface of the membrane and that K_1, the intrinsic sodium association constant, is 0.6 M^{-1} [24]. The addition of divalent cations to the bathing solution causes the zeta potential to become less negative. The dashed lines in Fig. 2 illustrate the theoretical prediction of the Stern equation if it is assumed that

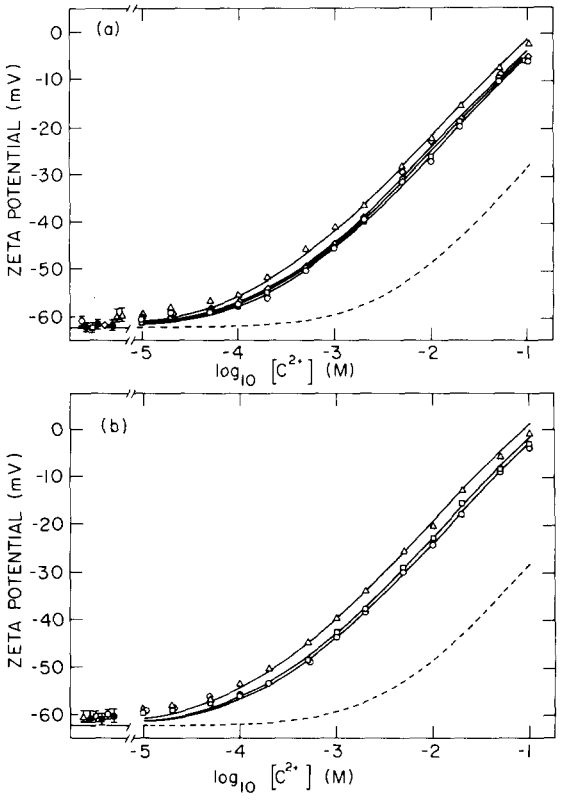

Fig. 2. The effect of divalent cations on the zeta potentials of multilamellar PG vesicles in 0.1 M sodium chloride. The solutions were buffered to pH 7.4 with 1 mM Mops, and also contained either 0.1 mM EDTA (filled symbols) or the indicated concentration of divalent cations (open symbols). T = 25°C. The points indicate the average of measurements on at least 20 vesicles in two separate experiments and the standard deviations shown at the left of the figure are typical values. The solid lines represent the least squares best-fits of the Stern equation (Eqns. 1, 2 and 6) to the data. The values of K_2, the intrinsic association constants (M^{-1}), obtained from these fits are: Mn^{2+}, 11.5; Ca^{2+}, 8.5; Ni^{2+}, 7.5; Co^{2+}, 6.0; Ba^{2+}, 5.5 and Sr^{2+}, 5.0. (a) Δ, Ca; ◇, Mg; □, Ba; ○, Sr. (b) Δ, Mn; □, Ni; ○, Co.

divalent cations do not adsorb to PG (i.e., K_1 = 0.6 M^{-1} and K_2 = 0 M^{-1}). All divalent cations produce a larger effect on the zeta potential than predicted by this screening curve. The simplest explanation is that the divalent cations both adsorb to and exert a nonspecific screening effect on PG membranes.

For each of the divalent cations illustrated in Fig. 2 the zeta potential reverses sign in the concentration range between 0.1 and 0.2 M (data not

shown). Eqns. 2 and 6 predict that the association constant for 1 : 1 complexes, K_2, is the reciprocal of the divalent cation concentration at which charge reversal occurs; thus the value of K_2 is between 5 and 10 M^{-1} for each of the divalent cations we examined. This determination of K_2 is independent of the degree of monovalent cation adsorption to the membrane, the number of 2 : 1 complexes formed with the divalent cation, the location of the plane of shear, the area the lipid occupies in the membrane and most of the assumptions inherent in the theory of the diffuse double layer.

This association constant can also be determined by fitting the Stern equation to the data illustrated in Fig. 2. The solid lines in Fig. 2 are the least squares fits of the Stern equation to the experimental data, assuming that the divalent cations form only 1 : 1 complexes with PG. The plane of shear is taken to be 2 Å from the surface and the value of K_1 to be 0.6 M^{-1} [24]. The values of K_2 range from 5 to 11.5 M^{-1}, in good agreement with the charge reversal measurements.

The Gouy-Chapman-Stern theory (Eqns. 1, 2 and 6) can also be used to predict the number of bound divalent cations [14]. In order to test this prediction we made independent measurements of the number of cations bound to PG. The number of bound calcium ions was determined by a calcium-sensitive electrode and the number of bound cobalt ions was determined from the ^{31}P-NMR linewidth (see below).

Electrode measurements of free calcium in sonicated PG vesicle solutions

Sonicated vesicles were formed in solutions containing 0.1 M sodium chloride, 1 mM Mops, pH 7.5. A known amount of calcium was added to the vesicle suspension after sonication and the free calcium concentration, $[Ca]^f$, was measured with a calcium-sensitive electrode (Neustra, Salt Lake City, UT). Under our experimental conditions the Gouy-Chapman-Stern theory predicts that the number of divalent cations in the diffuse double layer is negligible compared to the number of divalent cations bound to the membrane [2,34,35]. Therefore, we calculated the bound calcium concentration, $[Ca]^b$, from the expression:

$$[Ca]^b = [Ca]^{tot} - [Ca]^f \quad (7)$$

where $[Ca]^{tot}$ is the total calcium concentration. Assuming a 1 : 1 stoichiometry the apparent association constant, K_A, is defined as:

$$K_A = \frac{\{CaPG\}}{[Ca]^f\{PG\}} = \frac{[Ca]^b}{[Ca]^f[PG]} \quad (8)$$

where the braces denote a surface concentration, and [PG] is the concentration of PG molecules available to bind calcium ions. The value of [PG] was calculated from the measured value of the total PG concentration, $[PG]^{tot}$, and the expression:

$$[PG] = \frac{\alpha[PG]^{tot}}{(1 + K_1C^+ \exp(-F\psi_0/RT))} = 0.27[PG]^{tot} \quad (9)$$

The denominator in Eqn. 9 accounts for the postulated direct competition between sodium and calcium ions for the same binding site. K_1 is the intrinsic sodium-PG association constant, C^+ is the bulk sodium concentration and ψ_0 is the surface potential. K_1 was calculated to be 0.6 M^{-1} and ψ_0 was calculated to be −83 mV in 0.1 M sodium chloride [24]. The factor α, the fraction of PG molecules in the outer monolayer, was determined to be 0.67 (Lau, A. and McLaughlin, A.C., unpublished results).

The intrinsic association constant, K_2, is related to the apparent association constant, K_A, through the expression:

$$K_2 = K_A \exp(2F\psi_0/RT) \quad (10)$$

As expected, the calculated values of K_2 are independent of the total PG concentration (3 mM–6 mM) and the total calcium concentration (5 μM to 50 μM). The average value of K_2 for the assumed 1 : 1 complex between calcium and PG was 5.2 ± 0.7 M^{-1} (S.D., n = 45). This value agrees reasonably well with the value of the intrinsic association constant calculated from the electrophoresis experiments, 8.5 M^{-1} (see previous section).

^{31}P-NMR measurements of free cobalt in sonicated PG vesicle solutions

Figs. 3 and 4 illustrate the effects of cobalt on the ^{31}P-NMR linewidth of sonicated PG vesicles in 0.1 M sodium chloride.

As shown in Fig. 3, the ^{31}P-NMR linewidth,

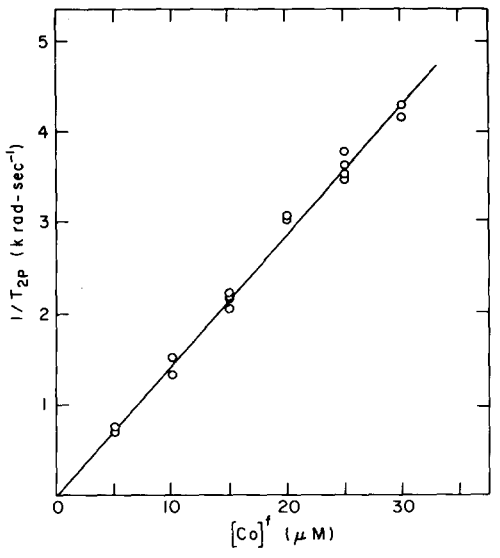

Fig. 3. The effect of cobalt on the ^{31}P-NMR linewidth of PG vesicles sonicated in 0.1 M sodium chloride. $1/T_{2P}$ is the calculated effect of cobalt on the linewidth of the ^{31}P-NMR signal from PG molecules in the outer monolayer of the vesicles and $[Co]^f$ is the free cobalt concentration in the sample.

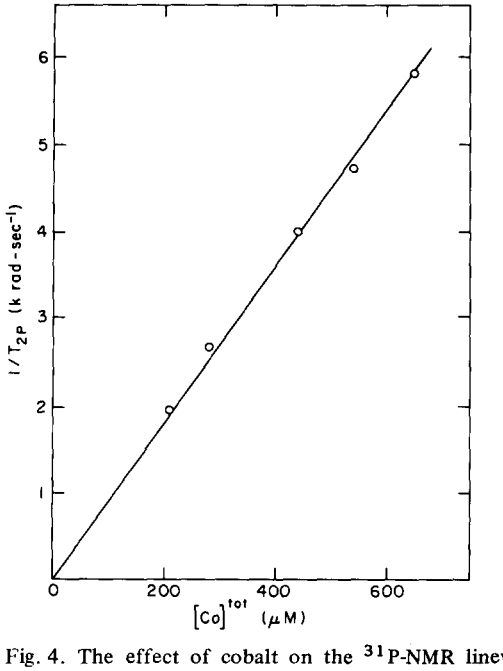

Fig. 4. The effect of cobalt on the ^{31}P-NMR linewidth of PG vesicles sonicated in 0.1 M sodium chloride. $1/T_{2P}$ is the effect of cobalt on the linewidth of the ^{31}P-NMR signal from the PG molecules in the outer monolayer of the vesicles and $[Co]^{tot}$ is the total concentration of cobalt.

which is proportional to the fraction of bound phosphate groups [11], is linearly dependent on the free cobalt concentration. This linear dependence implies that the number of bound cobalt ions was too small to affect the micropotential, the potential at the phosphate binding site.

For the experiments shown in Fig. 4, the free cobalt concentration in each sample, $[Co]^f$, was determined from the ^{31}P-NMR linewidth, using the solid line in Fig. 3 as a calibration curve. The bound cobalt concentration, $[Co]^b$, was calculated from the expression:

$$[Co]^b = [Co]^{tot} - [Co]^f \quad (11)$$

assuming that the amount of cobalt in the double-layer was negligible under these conditions (see above).

The apparent association constant for the cobalt-PG complex, K_A, is defined as (see Eqn. 8):

$$K_A = [Co]^b/[Co]^f[PG] \quad (12)$$

The intrinsic association constant, K_2, is related to the apparent association constant, K_A, by Eqn. 10. Assuming that $\psi_0 = -83$ mV [24] and using the values of $[Co]^f$ and $[Co]^b$ shown in Table I, the value of K_2 for the cobalt-PG complex was calculated to be 4.6 ± 0.2 M^{-1} (S.D., $n = 5$). As expected, the calculated value of K_2 was independent of the total cobalt concentration (200–650 μM). The value of K_2 agrees reasonably well with the value of the intrinsic association constant calculated from the electrophoresis experiments, 6.5 M^{-1} (see above).

^{13}C-NMR and ^{31}P-NMR determination of the cobalt-binding site in sonicated PG vesicles

The effects of cobalt on the ^{31}P-NMR spectrum of sonicated PG vesicles indicate that the phosphodiester group is involved in inner-sphere complexes with cobalt [10,11]. The ^{13}C-NMR spectrum of sonicated PG vesicles was used in an analogous manner to determine if other functional groups (i.e., the carbonyl or the hydroxyl groups) also form inner-sphere complexes with cobalt. The complete ^{13}C-NMR spectrum of sonicated PG vesicles is shown in the lower portion of Fig. 5. The signals between 15 and 35 ppm arise from carbon atoms in the fatty

TABLE I

^{31}P-NMR DETERMINATION OF INNER-SPHERE BINDING OF COBALT TO PG IN 0.1 M (A) AND 0.03 M (B) SODIUM CHLORIDE

The effect of cobalt on the ^{31}P-NMR line width of PG molecules in the outer monolayer ($1/T_{2P}$) was determined using the (π-τ-π/2) radio-frequency pulse sequence. The fraction of phosphate groups bound to cobalt in inner-sphere complexes, f, was calculated using Eqn. 13 assuming τ_M = 1.53 μs. The free cobalt concentration in the external aqueous medium, $[Co]^f$, was calculated from the ^{31}P-NMR linewidth using the solid lines in Figs. 3 and 9 as calibration curves. The bound cobalt concentration, $[Co]^b$, was calculated from Eqn. 11 and the bound PG concentration, $[PG]^b$, was calculated from Eqn. 14. The total PG concentrations were 21 ± 0.5 mM (A) and 24 ± 0.5 mM (B). The concentrations of PG on the outer monolayer were 14.3 ± 0.3 mM (A) and 18.7 ± 0.3 mM (B).

	$[Co]^{tot}$ (μM)	$1/T_{2P}$ (krad/s)	f (%)	$[Co]^f$ (μM)	$[Co]^b$ (μM)	$[PG]^b$ (μM)	$[PG]^b/[Co]^b$
A.	208	1.96	0.30	14	194	41	0.22
	274	2.67	0.41	18	256	57	0.22
	435	3.99	0.60	28	407	86	0.22
	540	4.71	0.72	33	507	98	0.19
	645	5.81	0.88	41	604	126	0.21
B.	220	1.69	0.26	2.2	217	48	0.22
	328	2.54	0.39	3.2	324	73	0.22
	435	3.15	0.48	3.9	431	90	0.21
	540	3.64	0.56	4.5	535	104	0.20

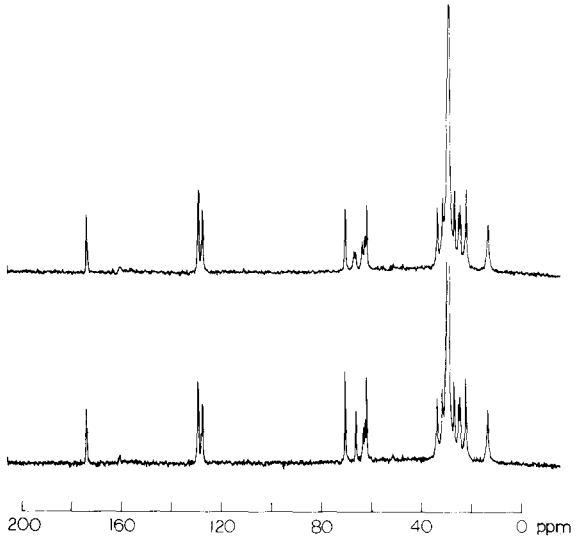

Fig. 5. ^{13}C-NMR spectra of sonicated PG vesicles in the absence (bottom) and presence (top) of 1 mM cobalt in the external aqueous medium. The signals between 62 and 72 ppm arise from carbon atoms in the polar head group, and are shown in more detail in Fig. 6. The ppm scale is downfield from tetramethylsilane. The solutions contained 0.1 M sodium chloride, 2 mM Mops, pH 7.5, and 20% ^2H_2O. The spectra were taken at 20°C with broad-band proton decoupling. The relatively broad signal for the terminal methyl group (15 ppm) is due to incomplete proton decoupling under these conditions.

acid chains, the two signals at 130 ppm arise from carbons in double bonds, and the two signals at 175 ppm arise from the carbonyl carbons. The ^{13}C-NMR spectrum of the polar head-group region is shown in more detail in the lower portion of Fig. 6. The assignments of the peaks are based on previous assignments for sonicated PC vesicles [36] and unpublished model studies. We note that the ^{13}C-NMR signal from the carbonyl groups (175 ppm) is split into two signals that are separated by approx. 0.44 ppm. This is very similar to the separation between the ^{13}C-NMR signals from carbonyl groups on the inside and outside monolayers of sonicated dipalmitoylphosphatidylcholine vesicles [37].

The addition of a relative high concentration of cobalt (1.0 mM) to the external aqueous solution had little effect on most of the signals in the ^{13}C-NMR spectrum (see upper portions of Figs. 5 and 6). The signals assigned to the carbonyl, double-bond and fatty-acid carbons were neither shifted nor broadened (Fig. 5). The lack of an observed shift in the signals assigned to the hydroxyl or carbonyl groups argues against the presence of an appreciable amount of inner sphere complexes of these ligands with cobalt. The only signals that clearly shift are those assigned

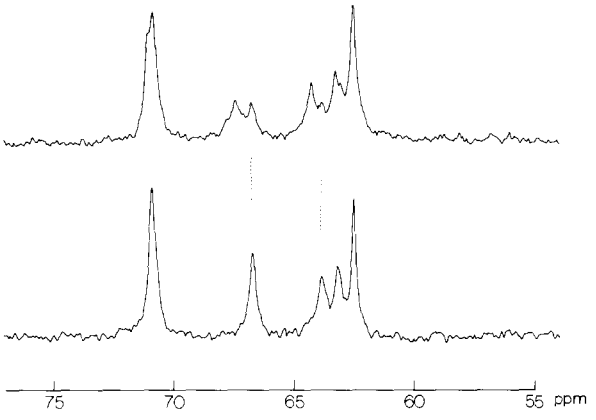

Fig. 6. The polar head group region of the ^{13}C-NMR spectrum of sonicated PG vesicles in the absence (bottom) and presence (top) of 1 mM cobalt in the external aqueous medium. The assignments are: 62.53 ppm, CH_2OH; 63.18 ppm, CH_2OR; 63.83 ppm, CH_2OP (polar headgroup); 66.74 ppm, CH_2OP (glycerol backbone); 70.95 ppm, CHOH and CHOR. R designates a fatty acid chain. The solutions contained 0.1 M sodium chloride, 0.2 mM Mops, pH 7.5 and 20% ^2H_2O. The spectra were taken at 20°C with broad-band proton decoupling.

to the two methylene groups adjacent to the phosphodiester group (see Fig. 6). The signals for the glycerol CH_2OP group and the choline CH_2OP group of PG molecules in the outer monolayer shifted 0.42 ppm downfield and 0.65 ppm downfield, respectively. These small shifts probably arise from the contact or pseudo-contact interaction of these carbons with cobalt ions bound to the adjacent phosphate group. The signals from the CH_2OP groups of PG molecules on the inner monolayer were unshifted (see dotted lines).

We conclude that only the phosphodiester group in the PG molecule forms detectable amounts of inner-sphere complexes with cobalt. Similar conclusions were also reached for PC (data not shown).

^{31}P-NMR measurement of the number of phosphate groups bound in inner-sphere complexes with cobalt

For the experimental data shown in Fig. 4 the linewidth of the ^{31}P-NMR signal for PG molecules on the outer monolayer, $1/T_{2P}$, is given by the expression [11]

$$1/T_{2P} = f/\tau_M \qquad (13)$$

f is the fraction of phosphate groups in the outer PG monolayer that are bound in inner-sphere complexes with cobalt, and τ_M is the lifetime of the complex [11], which is calculated to be 1.53 μs at 20°C (McLaughlin, A., unpublished results). f was calculated for each cobalt concentration shown in Fig. 4. The concentration of phosphate groups bound in inner-sphere complexes, $[PG]^b$, was then calculated from the expression:

$$[PG]^b = f \cdot \alpha [PG]^{tot} \qquad (14)$$

where $[PG]^{tot}$ is the total concentration of PG, and α accounts for the fraction of PG molecules in the outer monolayer ($\alpha = 2/3$). $[PG]^b$ was approximately one-fifth $[Co]^b$ for all the samples (Table I). If the stoichiometry of the inner-sphere complex of PG with cobalt is 1 : 1, only one-fifth of the bound cobalt ions are involved in inner-sphere complexes with phosphate groups. The ^{13}C-NMR results show that the remaining bound cobalt ions are not involved in inner-sphere complexes with other membrane ligands and a theoretical analysis indicates that they are not sequestered in the aqueous diffuse double layer. We conclude that the remaining cobalt ions are involved in outer-sphere complexes. In electrochemical terminology, these divalent cations would be considered part of the 'outer-Helmholtz layer' [38].

^{31}P-NMR measurement of the effect of calcium on the potential at the phosphate-binding site

Cobalt can also be used as a 'probe' to study the effect of diamagnetic divalent cations on the micropotential, ψ_m, the average potential at the phosphate-binding site. Very low cobalt concentrations (~20 μM) have a substantial effect on the ^{31}P-NMR linewidth of PG vesicles (Fig. 3) and this effect is proportional to the number of cobalt ions bound in inner-sphere complexes. The number of inner-sphere complexes is proportional to the free cobalt concentration in the aqueous phase adjacent to the phosphate group, and this free concentration is, in turn, proportional to the Boltzmann factor, $\exp(-2F\psi_m/RT)$. Because low cobalt concentrations affect neither the zeta potential (Fig. 2) nor the micropotential (Fig. 3) we were able to use the ^{31}P-NMR linewidth to measure changes in the micro-

potential produced by other cations such as calcium.

In the absence of cobalt the adsorption of calcium to PG membranes ([Ca] < 200 μM) does not significantly affect the ^{31}P-NMR linewidth. In the presence of cobalt the effect of calcium on the surface potential causes adsorbed cobalt ions to be displaced. Specifically, the bound calcium ions affect the micropotential, ψ_m, and the ratio of the ^{31}P-NMR linewidth in the presence, $1/T_{2P}^{Ca}$, and absence, $1/T_{2P}^{0}$, of calcium is given by the expression:

$$(1/T_{2P}^{Ca})/(1/T_{2P}^{0}) = \exp(-2F\Delta\psi_m/RT) \qquad (15)$$

where $\Delta\psi_m$ is the change in the micropotential on addition of calcium. The observed effect of calcium on the ^{31}P-NMR linewidth of sonicated PG vesicles in 20 μM cobalt chloride is shown in the left portion of Fig. 7. The values of $\Delta\psi_m$ calculated from Eqn. 15 are presented in the right portion of Fig. 7. The change in the surface potential calculated from the Gouy-Chapman-Stern theory using the intrinsic calcium association constant determined from electrode measurements with sonicated PG vesicles, $K_2 = 5.2$ M^{-1}, is illustrated by the dashed line in the right portion of Fig. 7. The changes in the micropotential determined from the NMR measurements are very similar to the changes in the surface potential predicted from the Gouy-Chapman-Stern theory.

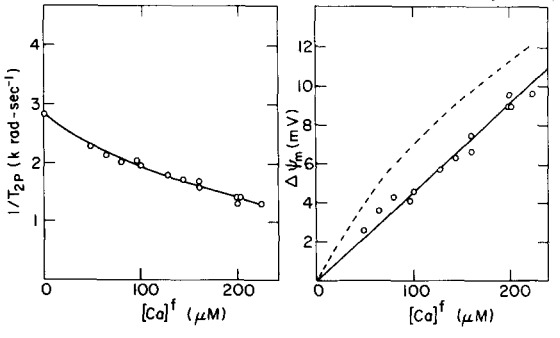

Fig. 7. The effect of calcium on the ^{31}P-NMR linewidth (left) and the micropotential (right) of sonicated PG vesicles in the presence of 20 μM free cobalt. $1/T_{2P}$ is the ^{31}P-NMR linewidth of PG molecules in the outer monolayer of the PG vesicles and $[Ca]^f$ is the free calcium concentration in the external aqueous medium. The dashed line is the change in the surface potential predicted by the Stern equation (see text). $\Delta\psi_m$ is the change in the micropotential, the potential at the phosphate binding site. Each point in the right graph was determined from the corresponding point on the left graph using Eqn. 15.

Zeta potential measurements in 0.01 M sodium chloride

The Gouy-Chapman-Stern theory predicts that a decrease in the sodium chloride concentration from 0.1 M to 0.01 M should decrease the zeta potential of PG vesicles from −62.5 mV to −118 mV. The observed zeta potentials are −60 mV and −110 mV (see Figs. 2 and 8), in reasonable agreement with this prediction. The theory also predicts that the calcium concentration required to produce a measurable effect on the zeta potential should decrease by two orders of magnitude. The results presented in Figs. 2 and 8 qualitatively confirm this prediction. For example, in 0.1 M sodium chloride a calcium concentration of 3 mM is required to produce a 25 mV

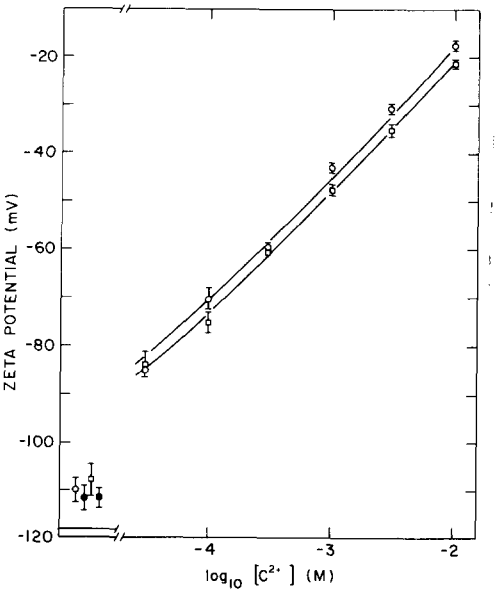

Fig. 8. The effect of calcium (○) and cobalt (□) on the zeta potentials of multilamellar PG vesicles in 0.01 M sodium chloride. T = 25°C. The solutions, which were buffered with 1 mM Mops to pH 7.4, also contained either 0.1 mM EDTA (filled symbols) or the indicated concentration of divalent cations (open symbols). The points represent the average (+S.D.) of measurements on at least 20 vesicles in two separate experiments. The solid lines represent the least-squares fits of the data to the Stern equation (Eqns. 1, 2 and 6) which were obtained with the value of K_2 = 17 M^{-1} for calcium and K_2 = 13 M^{-1} for cobalt.

change in the zeta potential, while in 0.01 M sodium chloride a calcium concentration of 30 μM produces approximately the same effect. However, the intrinsic association constants calculated from the data obtained in 0.01 M sodium chloride (Fig. 8) are a factor of two larger than the values calculated from the data obtained in 0.1 M sodium chloride (Fig. 2). The cause of this deviation from the prediction of the Gouy-Chapman-Stern theory is not apparent, although similar deviations have been observed for PS and PC membranes [2,11,39].

^{31}P-NMR measurements in 0.03 M sodium chloride

The Gouy-Chapman-Stern theory predicts that the apparent cobalt association constant should increase with a decrease in sodium chloride concentration. The experiments shown in Figs. 9 and 10 are similar to those shown in Figs. 3 and 4, except that the solution contained 0.03 M instead of 0.1 M sodium chloride. K_2 for the cobalt-PG complex was calculated to be 2.2 + 0.2 M^{-1} (S.D., $n = 4$). This value of K_2 is approximately one-half the value calculated from the data in 0.1 M sodium chloride, 4.6 + 0.2 M^{-1} (see above). The deviation is in the opposite direction to that found for the values of K_2 calculated from the zeta potential data. The cause of these deviations is unknown.

For each cobalt concentration $[PG]^b$ is approximately one-fifth $[Co]^b$ (see Table I). Similar results were obtained in 0.1 M sodium chloride (see Table I). We conclude that in both 0.03 and 0.1 M sodium chloride only one-fifth of the bound cobalt ions are involved in inner-sphere complexes with the phosphate group.

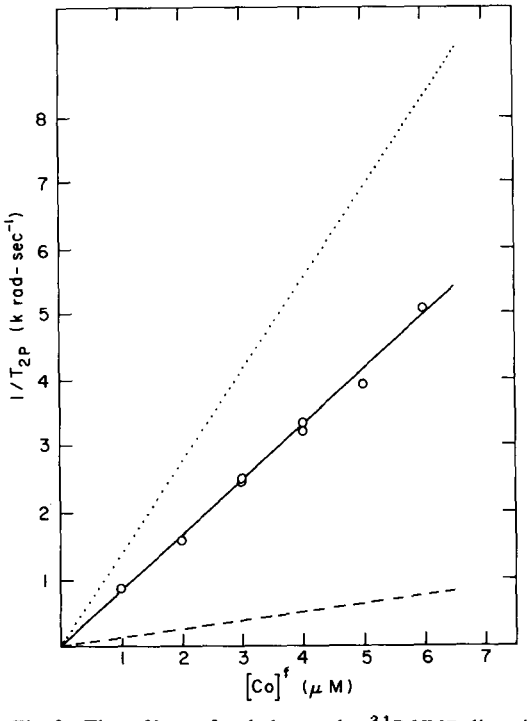

Fig. 9. The effect of cobalt on the ^{31}P-NMR linewidth of sonicated PG vesicles in 0.03 M sodium chloride. $1/T_{2P}$ is the calculated effect of cobalt on the linewidth of the ^{31}P-NMR signal from PG molecules in the outer monolayer of the sonicated vesicles and $[Co]^f$ is the free cobalt concentration in the sample. The solid line is the best linear fit to the data. The dashed line represents the experimental data from a similar experiment in 0.1 M sodium chloride (Fig. 3) and the dotted line represents the prediction of the Stern equation (Eqns. 1, 2 and 6) for 0.03 M sodium chloride.

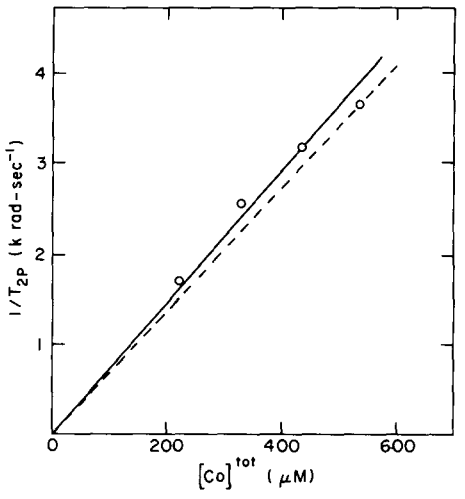

Fig. 10. The effect of cobalt on the ^{31}P-NMR linewidth of sonicated PG vesicles in 0.03 M sodium chloride. $1/T_{2P}$ is the linewidth of PG molecules in the outer monolayer and $[Co]^{tot}$ is the total cobal concentration in the external aqueous medium. Other details are given in the legend for Fig. 4. The solid line is the best linear fit to the data in 0.03 M sodium chloride and the dashed line represents the data from a similar experiment in 0.1 M sodium chloride (see Fig. 4).

Discussion

Our major conclusion is that the simple Gouy-Chapman-Stern theory adequately describes the adsorption of divalent cations to bilayer membranes formed from PG in 0.1 M sodium chloride.

The theory relates the surface potential to the concentration of divalent cations in the bulk aqueous medium in terms of a single adjustable parameter, the intrinsic association constant of these cations with the phospholipid molecules. The surface potential cannot be measured directly, so we determined the zeta potential, the potential at the hydrodynamic plane of shear, from the electrophoretic mobility of the vesicles. From Fig. 2, it is seen that the theory provides a good description of the ζ potential data.

The Gouy-Chapman-Stern theory also relates the number of adsorbed divalent cations to the concentration of divalent cations in the bulk aqueous medium. This relationship was tested experimentally by following the binding of calcium to PG vesicles with a calcium-sensitive electrode and by studying the effects of cobalt on the ^{31}P-NMR spectrum of these vesicles. The electrode measurements indicate that the intrinsic association constant for the calcium-PG complex is 5.2 M^{-1}, while the value determined from the zeta potential data is 8.5 M^{-1}. The ^{31}P-NMR results indicate that the intrinsic association constant for the cobalt-PG complex is 4.6 M^{-1}, while the value determined from the zeta potential data is 6.5 M^{-1}. The observed agreement is considered to be satisfactory.

In the Gouy-Chapman-Stern theory it is assumed that the charges on the phospholipid molecules and the bound divalent cations are uniformly 'smeared' over the surface of the membrane. This assumption is not true. On a negatively charged membrane the magnitude of the 'micropotential', the average potential at the divalent cation binding site, is larger than the average value of the surface potential. Furthermore, when divalent cations bind to a small fraction of the phospholipid molecules, they exert a smaller effect on the micropotential than on the average value of the surface potential. These phenomena are usually termed 'discrete-charge' effects [40–44]. We measured the effect of calcium on the micropotential of PG membranes and compared these results with the calculated effects of calcium on the average value of the surface potential. The changes in the micropotential were assumed to be equivalent to the changes in the potential at the phosphate group, which we have shown is the divalent cation binding site in PG membranes. The changes in the average value of the surface potential were calculated from the Gouy-Chapman-Stern theory. The agreement between the measured changes in the micropotential and the calculated changes in the average value of the surface potential on the addition of calcium is surprisingly good (Fig. 5). The observation that the changes in the micropotential are slightly less than the calculated changes in the average value of the surface potential is consistent with a small discrete-charge effect.

The Gouy-Chapman-Stern theory qualitatively accounts for the effects of monovalent ions on the surface potential and, therefore, on the apparent divalent cation association constant (Eqn. 10). However, the good quantitative agreement observed between the intrinsic association constants determined by the fit of the Gouy-Chapman-Stern theory to the zeta potential data and the association constants determined from the direct ^{31}P-NMR measurements in 0.1 M sodium chloride solutions is not found at the lower sodium chloride concentrations. Given the simplicity of the model, this minor discrepency is not surprising. Rather, it is surprising that the Gouy-Chapman-Stern theory explains the zeta potential and direct adsorption measurements in 0.1 M sodium chloride as well as it does.

The paramagnetic effects of cobalt on the ^{31}P- and ^{13}C-NMR spectra of PG membranes were used to determine which groups bind cobalt ions. We conclude that only the phosphodiester group forms inner-sphere complexes with cobalt, but that only about one-fifth of the bound cobalt ions are involved in inner-sphere complexes. The remainder of the bound cobalt ions are involved in outer-sphere complexes. Outer-sphere complexes, which were first proposed by Werner in 1913 [45], are envisioned as ion-pairs formed by the electrostatic attraction between the fully hydrated divalent cation and the negative ligand [3,4]. The outer-sphere complexes of cobalt with the PG molecules presumably involve an ion-pair with the phosphodiester group.

Our conclusion that a substantial fraction of bound cobalt ions are involved in outer-sphere com-

plexes with PG is consistent with previous studies of cobalt binding to model oxy-anions. For example, Eigen demonstrated that sulphate ions form predominantly outer-sphere complexes with cobalt using ultrasound absorption techniques [46]. Williams came to the same conclusion for both sulphate and nitrate ions using spectroscopic techniques [47]. Finally, Brintzinger concluded that approximately one-half of the terminal phosphate groups of CoATP were involved in outer-sphere complexes with cobalt using infrared techniques [48].

Although we have no direct experimental evidence that the other divalent cations used in this study also form mainly outer-sphere complexes with PG, three circumstantial lines of evidence are consistent with this suggestion. First, Phillips [49] pointed out that when an anion forms predominantly outer-sphere complexes with a series of divalent cations, the association constants are usually very similar [46,50]. The association constants for the PG complexes of the alkaline earth and divalent transition metal ions we investigated are very similar. Second, the association constant for this type of complex is predicted theoretically to be of order 10 M^{-1} [3,4], which is similar to the intrinsic association constants found for PG. Third, many of the alkaline earth and divalent transition metal ions studied here are known to form outer-sphere complexes with oxy-anions such as sulphate [46] or the terminal phosphate group of ATP [48], and Puskin has suggested that manganese forms outer-sphere complexes with the phosphate groups of cardiolipin [34].

The effects of divalent cations on the zeta potentials of PG membranes are comparable to those observed previously with phosphatidylserine (PS) membranes [2]. For example, the intrinsic association constants for the Ca-PS (12 M^{-1}) and Mg-PS (8 M^{-1}) complexes are similar to those for the Ca-PG (8.5 M^{-1}) and Mg-PG (6 M^{-1}) complexes. These intrinsic association constants, obtained with the negative lipids PS and PG, are also similar to the intrinsic association constants obtained with the zwitterionic lipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [2]. Thus, of the preponderant lipids in the plasma membranes of most plant and animal cells, none display much selectivity in their complexes with the alkaline earth cations and the association constants are approximately 1–10 M^{-1}. This observation is consistent with the hypothesis that the alkaline earth cations form predominantly outer sphere complexes with these four lipids.

Acknowledgements

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