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THE ADSORPTION OF DIVALENT CATIONS TO PHOSPHATIDYLCHOLINE BILAYER MEMBRANES

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Summary

Electrophoretic mobility and ^{31}P NMR measurements were combined to test whether the combination of the Henry, Boltzmann and Grahame equations is capable of describing the adsorption of divalent cations to phosphatidylcholine membranes. Cobalt was chosen for this study because, of all the common divalent cations, its effects on the ^{31}P NMR spectrum of phosphatidylcholine membranes are easiest to interpret. Both the ^{31}P NMR data on the adsorption of cobalt and the zeta potential data calculated from the electrophoretic mobility in the presence of cobalt are well described by the combination of these three equations. Electrophoretic mobility measurements were also performed with a number of other divalent cations and the zeta potentials were, in all cases, well described by the combination of these three equations. The binding deduced from such measurements decreases in the sequence: Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Sr^{2+}, Ba^{2+}. If we assume that a lipid molecule occupies an area of 60 $Å^2$ and that there is a 1 : 1 stoichiometry for the binding of the divalent ions to phosphatidylcholine, the dissociation constants are, respectively: 0.3, 1.0, 1.0, 1.2, 1.2, 2.8, 3.6 M.

Introduction

Divalent cations affect biological membranes in many ways. Although a number of these effects probably arise from specific binding to membrane proteins, some of the effects may arise from a more general interaction with the membrane surface. Calcium, for example, has long been known to 'stabilize' the electrically excitable membranes of nerve and muscle; in the presence of an increased concentration of calcium in the extracellular fluid the membrane

must be depolarized to a greater degree to elicit an action potential. The voltage clamp studies of Frankenhaeuser and Hodgkin [1] clearly demonstrated that this stabilizing effect was due to the ability of calcium to shift the conductance-voltage curves of the nerve along the voltage axis. Huxley [1] suggested that calcium produced this shift by adsorbing to the outer surface of the membrane, creating a positive surface potential. A positive potential at the outer surface of the membrane would produce a larger drop in the potential within the membrane. The potential difference which can be measured by electrodes in the bulk aqueous phases, essentially a diffusion potential due to the exit of potassium ions from the cell, remains, as one would expect theoretically, unchanged by calcium. Gilbert and Ehrenstein [2] and McLaughlin et al. [3] modified the hypothesis slightly by suggesting that calcium and other divalent cations could decrease the magnitude of the potential at the outer surface of the nerve by 'screening' existing negative charges on the membrane as well as by binding to specific sites. The term 'screening' is used to connote the existence of ions a few angstroms from the surface of the membrane in the aqueous diffuse double layer and to distinguish this effect from the phenomenon of specific adsorption. In the last few years, results have accumulated from experiments on excitable biological membranes which strongly support this modified hypothesis [4—11]. The work of Hille et al. [10] is particularly convincing because these investigators have shown that monovalent as well as divalent cations are capable of producing shifts in the conductance-voltage curves, as predicted by the theory of the diffuse double layer

Artificial phospholipid monolayers and bilayers are good model systems for testing the applicability of the diffuse double layer theory to biological interfaces. Many independent studies have confirmed that the electrostatic potentials at the surfaces of negatively charged bilayers and monolayers do vary in the predicted manner with changes in the charge density and the concentrations of both monovalent and divalent cations in the bulk solutions (for reviews refs. 12 and 13), Until recently, however, it has not been possible to measure the adsorption of divalent cations to the polar head groups of lipids. Classical techniques (e.g. measurements of the uptake of radioactive calcium beneath a monolayer, equilibrium dialysis measurements on bilayer vesicles) are not suitable for this purpose because they do not distinguish between ions actually adsorbed to the surface of a negatively charged membrane and ions merely confined to the aqueous diffuse double layer. They are not sensitive enough, furthermore, to measure the relatively weak adsorption of ions as such as calcium to zwitterionic lipids, the topic of this report.

To describe the adsorption of divalent cations to phosphatidylcholine bilayers one must recognize that the adsorption produces a positive electrostatic potential at the surface of the membrane. This positive potential will lower the concentration of the adsorbing cations in the aqueous phase immediately adjacent to the membrane. The simplest theoretical relation between the number of adsorbed divalent cations, the surface potential and the concentration of cations in the bulk aqueous phase is provided by a combination of the Boltzmann relation, the Grahame equation from the theory of the diffuse double layer, and a Henry law adsorption isotherm.

Grasdalen et al. [14] and Hutton et al. [15] have recently measured shifts in

the ^{31}P NMR signals of phosphatidylcholine upon addition of calcium. Grasdalen et al. argued that their results were consistent with the combination of the above three equations and the zeta potential measurements reported here support this claim. ^{31}P NMR measurements with calcium do not, however, provide a satisfactory test of these equations because the shifts produced by calcium are very small and no calibration factor is available to relate the observed shift to the fraction of bound phosphate groups.

In this article we attempt to circumvent these problems by using the divalent cation cobalt. Cobalt was chosen for these studies because it is paramagnetic and, in contrast to calcium which is diamagnetic, has very large effects on the ^{31}P NMR spectrum of phospholipid bilayer membranes. The effects of cobalt on the ^{31}P NMR spectrum of phosphodiester groups are, furthermore, well understood theoretically [9,16,17]; by calibrating the effects with a model compound, glycerophosphoryl choline, we can calculate the fraction of phosphate groups in phosphatidylcholine bound to cobalt. To test whether the combination of the Boltzmann, Grahame and Henry equations is capable of quantitatively describing the adsorption of divalent cations to membranes we combined electrophoretic mobility and 'probe' estimates of the surface potential with ^{31}P NMR measurements.

Materials and Methods

Estimates of the surface potential

The vesicles for the microelectrophoresis experiments were prepared from either egg phosphatidylcholine (Avanti, Lipid Products, Supelco, and a sample kindly supplied by M. Eisenberg) or dioleoyl phosphatidylcholine (Supelco). Identical results were obtained with all samples and the results were pooled for the final analysis. The phosphatidylcholine was dissolved in spectroscopic grade chloroform and methanol, dried in a rotary evaporator, then a solution containing 0.1 M NaCl buffered to pH 7.5 with 0.01 M Tris-HCl was added. Multilamellar dispersions of the appropriate size (1—10 μm) for mobility measurements were prepared by adding a few glass beads and gently shaking the flask [18]. The appropriate divalent cation was added as the chloride salt (Baker or Fisher), then measurements of the electrophoretic mobility were made at 25 ± 1°C on a commercially available machine (Rank Bros., U.K.) based on a design by Bangham et al. [19]. Care was taken to focus at the stationary layer. The zeta potential, ζ, or potential at the hydrodynamic plane of shear was calculated from the Helmholtz-Smoluchowski equation: $\zeta = \eta u/\epsilon_r\epsilon_o$ where u is the mobility, η is the viscosity, ϵ_o is the permittivity of free space and ϵ_r is the dielectric constant of the aqueous solutions. The assumptions inherent in the derivation of this equation are discussed elsewhere [20,21].

In two recent studies the zeta potentials of lipid dispersions were compared with the measured values of the charge density [22,23]. Good agreement with the Gouy equation from the theory of the diffuse double layer was observed in all cases. For low charge densities the plane of shear is thought to lie within a few angstroms of the envelope of the head groups. The experimentally determined zeta potentials reported here should, therefore, provide a reasonable approximation to the electrostatic potential at the surface of the bilayer, ψ, the

parameter which appears in the theoretical equations below.

To confirm this expectation, we measured the change in electrostatic potential which occurred in the middle of a phosphatidylcholine membrane when a divalent cation was added to the aqueous phases. We chose calcium for this purpose. To monitor the change in potential in the middle of the membrane we measured the anion and cation conductances produced, respectively, by 5,6-dichloro-2-trifluoromethylbenzimidazole (DTFB) and by nonactin. The use of conductance measurements to deduce changes in electrostatic potentials within membranes is discussed elsewhere [24,12]. The membranes were formed from diphytanoyl phosphatidylcholine (Avanti) in 0.1 M NaCl buffered to pH 7.5 with 0.01 M Tris at 22°C. The solutions for the cationic probe measurements also contained 0.01 M KCl. We checked that the lipid did not contain negatively charged contaminants by making multilamellar dispersions and confirming that their electrophoretic mobilities were zero. We also agitated the membrane-forming solution (10 mg/ml of lipid in decane) with the aqueous solution. The zeta potentials of these monolayer-coated decane droplets were also zero. The planar black lipid membranes were formed using standard techniques and the conductances of the membranes were measured in the presence of the carrier. Calcium was then added to both bathing solutions and the change in conductance noted. The membranes were aged 20 min before each experiment and measurements were only accepted when a single membrane lasted for an entire experiment because the changes in conductance were quite small.

Estimates of the fraction of phosphate groups bound to cobalt

(a) Variation of temperature and magnetic field strength at constant cobalt concentration. In order to calibrate the observed ^{31}P NMR linewidth in terms of the fraction of bound phosphate groups the value of τ_M, the lifetime of the phosphate group in the first coordination sphere of cobalt, must be determined. To do this the temperature and magnetic field strength dependence of the effects of cobalt on the ^{31}P NMR spectrum were investigated (see Appendix). Egg-yolk lecithin (Grade I) was obtained from Lipid Products, South Nutfield, U.K., and was used without further purification. Multilamellar dispersions formed in 0.1 M NaCl from the batch of lipid used in these experiments had zeta potentials of 0 mV. The sonicated vesicles for the ^{31}P NMR experiments were prepared in 99.9% deuterium oxide containing 0.1 M NaCl, 0.006 M $CoCl_2$ and 0.01 M Tris at pH 7.5. The sonication was performed in an ice-bath under a stream of nitrogen with a probe-type sonicator (Dawes) using a 30% duty cycle. The vesicles were sonicated for a total of 4—6 min, during which time the solution became clear. The vesicles were then centrifuged to remove titanium. Since the size of the vesicles does not substantially affect the observed line-width in the presence of 0.006 M $CoCl_2$, the sonicated vesicle preparation was not fractionated. After centrifugation the sample was divided into two aliquots for measurements in the different spectrometers. The two NMR spectrometers employed in these experiments were a Bruker WH-90 and an instrument constructed in the Biochemistry Department at Oxford operating at 129 MHz on phosphorus. Both instruments were interfaced with Nicolet 1080 computers, were operated in the Fourier transform mode and were equip-

ped with variable temperature accessories which maintained the temperature constant to ±1°C. Both spectrometers employed a deuterium 'lock'.

The temperature variations were performed by raising the temperature by 10°C steps and, after offsetting the temperature by 5°C, lowering the temperature in 10°C steps. This procedure would have given an indication of any irreversible phenomena, such as aggregation or precipitation. Neither time-dependent effects on the linewidths nor precipitation was observed over the period of the experiments (approx. 8 h).

(b) Variation of cobalt concentration at constant temperature and magnetic field strength. Egg-yolk lecithin was obtained from Sigma Chemical Company (type V-E), Avanti Biochemicals Inc., or was kindly supplied by M. Eisenberg. The samples from M. Eisenberg or Sigma were used for the experiments in 0.1 M NaCl while the samples from Avanti were used for the experiments in 1 M NaCl. Multilamellar dispersions formed from all these samples in 0.1 M NaCl had zeta potentials of 0 mV, within experimental error. The sonication procedure was identical to that given above except that a Branson model W185D sonicator was employed and a longer (12 min) continuous sonication at low power was used. Multilamellar dispersions formed from lipids extracted from the sonicated samples had zeta potentials of less than 2 mV in magnitude.

A Bruker WH-360 spectrometer operating at 145 MHz on phosphorus was employed in these experiments. The spectrometer was interfaced with a Nicolet 1180 computer and was equipped with a Bruker variable temperature accessory which maintained the temperature constant to within ±1°C. The titration of the ^{31}P NMR linewidth as a function of cobalt concentration was performed at 20°C. Both lipid samples gave identical results. At this temperature and magnetic field strength the fraction of phosphate groups bound to cobalt, f, may be estimated from the equation (see Appendix) $1/T_{2P} = f/\tau_M$, where $1/T_{2P} = \pi\Delta\nu$ and $\Delta\nu$ is the observed linewidth of the ^{31}P NMR signal. τ_M is the lifetime of the phosphate groups in the first coordination sphere of the cobalt ions. From the temperature and magnetic field dependence of the ^{31}P NMR signal at a constant cobalt concentration (see Appendix) the value of τ_M at 20°C was calculated to be $3.0 \cdot 10^{-6}$ s.

Theory

Both a Langmuir and a Volmer adsorption isotherm reduce to Henry's law when the fraction of bound sites is much less than unity [20]. A Henry's law adsorption isotherm is appropriate in our case because cobalt is bound to less than 5% of the phosphatidylcholine molecules (Fig. 4). The number of adsorbed divalent ions per unit area of membrane, Γ, is half the charge density, σ, which we assume to be linearly related to the concentration of divalent ions in the aqueous phase at $x = o$, the membrane-solution interface, $C^{2+}(0)$:

$$\Gamma = \frac{\sigma}{2} = KC^{2+}(0) \tag{1}$$

The constant K is discussed below and an interpretation in terms of statistical mechanics is given by Aveyard and Haydon [20]. The Boltzmann equation

relates the interfacial concentration to the bulk concentration $C^{2+}(\infty)$ by means of the surface potential, ψ:

$$C^{2+}(0) = C^{2+}(\infty) \exp(-2F\psi/RT) \tag{2}$$

The Grahame equation [12,20] from the theory of the diffuse double layer relates the surface potential to the charge density and the bulk aqueous concentration of monovalent and divalent ions, $C^i(\infty)$:

$$\sigma = \{2\epsilon_r\epsilon_0 RT \sum_i C^i(\infty)[\exp(-z_i F\psi/RT) - 1]\}^{1/2} \tag{3}$$

where ϵ_o is the permittivity of free space and ϵ_r is the dielectric constant of water.

Many assumptions enter into Eqns. 1—3. These assumptions have been discussed many times in the past, and the validity of these three equations has been tested and found to describe quite accurately the hydrophobic adsorption of monovalent anions and cations to bilayer membranes [12,13]. We are dealing here, however, with divalent rather than monovalent ions and with specific adsorption rather than non-specific hydrophobic adsorption. It is not unreasonable to suspect that the discrete charge effect, for example, could be more important when dealing with the adsorption of these divalent cations. Note that Eqns. 1—3 can be combined to eliminate either σ or ψ. We refer to both of these relations as Stern equations. To test experimentally the ability of the Stern equations to describe the adsorption both the surface potential and the number of bound phosphate groups were measured.

In a similar analysis of the adsorption of calcium to phosphatidylcholine membranes, Grasdalen et al. [14] assumed that chloride ions could also adsorb to the membranes. We know of no evidence that either sodium or chloride can bind to phosphatidylcholine vesicles and we will assume that such binding is negligible here *.

If the lipid molecules formed only 1 : 1 complexes with cobalt, the resulting surface concentration of divalent cations (number of adsorbed divalent cations per unit area), Γ_1, would be:

$$\Gamma_1 = K_1 C^{2+}(0) \tag{4}$$

and the fraction of phosphate groups bound to cobalt, f_1, would be:

$$f_1 = \Gamma_1 \cdot b \tag{5}$$

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* Our unpublished electrophoretic mobility measurements, which confirm and extend those of Hanai et al. [25], demonstrate that in NaCl solutions ranging from 0.001 to 0.5 M, the zeta potentials of phosphatidylcholine vesicles are zero within experimental error. This result is admittedly consistent with the unlikely possibility that both sodium and chloride ions adsorb to the same degree to phosphatidylcholine vesicles, but there is some NMR evidence which argues against this possibility. The observations of Jendrasiak [26] demonstrate that when anions such as thiocyanate, which do adsorb weakly to phosphatidylcholine bilayers [27,28], are added to phosphatidylcholine vesicles to a concentration of 0.1 M they produce a shift in the $N^+(CH_3)_3$ signal of the exterior head groups. Chloride ions, at the same concentration, had no effect on the head group signal. These measurements are consistent with our observation that the zeta potentials of phosphatidylcholine vesicles in NaCl solutions are zero, and we interpret these electrophoretic mobility measurements to mean that the dissociation constant of chloride ions with phosphatidylcholine is greater than 40 M. (The vesicles would have a zeta potential of —2 mV in a 0.5 M solution if the dissociation constant were 40 M).

where b is the area per lipid molecule, which we assume is 60 Å^2 [29], and K_1 is a constant. On the other hand, if the lipid molecules formed only 2 : 1 complexes with cobalt, the resulting surface concentration of divalent cations, Γ_2, would be:

$$\Gamma_2 = K_2 C^{2+}(0) \tag{6}$$

and the fraction of phosphate groups bound to cobalt, f_2, would be:

$$f_2 = 2\Gamma_2 \cdot b \tag{7}$$

where K_2 is a constant. If both 1 : 1 and 2 : 1 complexes are present, we assume that the total surface concentration of divalent cations, Γ, and the total fraction of bound phosphate groups, f, are simply $\Gamma_1 + \Gamma_2$ and $f_1 + f_2$, respectively. It follows, from Eqns. 1, 4 and 6 that:

$$K = K_1 + K_2 \tag{8}$$

and from Eqns. 4, 5, 6 and 7 that:

$$f = kC^{2+}(0) \tag{9}$$

where

$$k = (K_1 + 2K_2) \cdot b \tag{10}$$

Eqns. 1—3 may be combined to eliminate σ and yield an expression for ψ as a function of $C^{2+}(\infty)$. Fitting the zeta potential data obtained from the microelectrophoresis measurements to this expression yields a value for K. Once K has been determined, Eqns. 1, 2, 3 and 9 may be combined to yield an expression for f as a function of $C^{2+}(\infty)$. Fitting the f data obtained from the ^{31}P NMR experiment to this expression yields a value for k. Once k and K are known, the values of K_1 and K_2 may be determined from Eqns. 8 and 10 and these numbers define the amount of lipid in each type of complex. In essence, we are using the zeta potential data to determine the number of bound cobalt ions and the ^{31}P NMR data to determine the number of bound phosphate groups: the combination of the two numbers gives the effective stoichiometry. It is not necessary to assume a specific adsorption model to determine the fraction of lipid bound to cobalt in 1 : 1 and 2 : 1 complexes. To express our results in terms of conventional dissociation constants we make the following assumptions.

For the 1 : 1 complex we assume that each phosphate group forms an independent binding site. The surface area per binding site is then b, the surface area per lipid molecule. The surface density of binding sites is simply $1/b$. The dissociation constant for the 1 : 1 complex, K_{D1}, is then given by the expression:

$$K_{D1} = 1/(b \cdot K_1) \tag{11}$$

where K_1 is defined in Eqn. 4.

The treatment of the 2 : 1 complex is not so straightforward *. While the sur-

* We express the number of 2 : 1 binding sites and 2 : 1 complexes in terms of conventional surface concentrations. As we deal only with very low binding levels, the free concentration of sites is approxi-

face area per binding site is $2b$, the effective surface density of binding sites may be larger than $1/2b$ because of statistical factors. For simplicity, we make the conventional [31] assumption that the surface density of binding sites is $1/2b$. The dissociation constant for the 2 : 1 complex, K_{D2}, is then given by the following expression:

$$K_{D2} = 1/(2bK_2) \tag{12}$$

Results

Fig. 1 illustrates the effect of the divalent cations manganese, magnesium, nickel, strontium and barium on the zeta potentials of unsonicated phosphatidylcholine vesicles. Note that the zeta potentials were all initially zero. This indicates that the phosphatidylcholine did not contain a significant percentage of charged contaminants **. The addition of divalent cations produced an increase in the zeta potential, which in all cases could be well described by the combination of Eqns. 1—3, a combination we refer to as a Stern equation. Recall that there is only one adjustable parameter in this equation, the value of the constant K.

In Fig. 2 the zeta potential data are illustrated for cobalt and calcium. The data may again be well described by the Stern equation. Note that cobalt and calcium have similar binding constants.

To test that the zeta potential, the potential measured at the hydrodynamic plane of shear, is a reasonable approximation to the average electrostatic potential sensed by the divalent cations at the surface of the membrane, we measured the effect of calcium on the carrier mediated conductance of phosphatidylcholine black lipid membranes. It is apparent from Table I that calcium produces an increase in the conductance of an anion selective membrane (DTFB)

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mately equal to the total concentration of sites. If one wished to define an equilibrium 2 : 1 binding constant which depended quadratically on the surface concentration of lipids, our binding constants would be changed only by a constant factor. Hauser et al. [30] on the other hand, defined a binding constant for 2 : 1 complexes in terms of a volume concentration of lipids. This leads to contradictions. For example, equation 1 in their paper predicts that the number of 2 : 1 complexes will depend quadratically on the volume concentration of lipids when the system is far from saturation. This is obviously not true if the 2 : 1 complex is formed between a metal ion and two lipids in the same vesicle. Their formulation would be correct if the complexes were formed between lipids on two different vesicles and a metal ion.

** The standard deviations are about 1 mV (Fig. 1). This implies, via the Grahame or Gouy equation, that negatively charged contaminants constitute less than 0.3% of the lipids. By making measurements at lower electrolyte concentrations we confirmed that the contaminant level was actually much less than this. We note in passing that microelectrophoresis appears to be one of the most sensitive methods available for detecting charged contaminants in zwitterionic or neutral lipids. When measurements are made in a solution of low salt concentration, it is at least an order of magnitude more sensitive than thin layer chromatography. We used the electrophoresis technique to check that the ultrasonic irradiation of egg PC samples did not introduce a significant percentage of negatively charged contaminants [32]. After sonication we extracted the lipids into either chloroform or chloroform : methanol (2 : 1), resuspended the lipids in 0.1 M NaCl in the form of large multilamellar vesicles, and measured the zeta potentials. The zeta potentials were lower in magnitude than —2 mV, which implies, by means of the Gouy equation, that less than 0.6% negative contaminants were introduced by the sonication procedure used to prepare vesicles for the NMR experiments. The sonication was carried out under nitrogen atmosphere at 0°C.

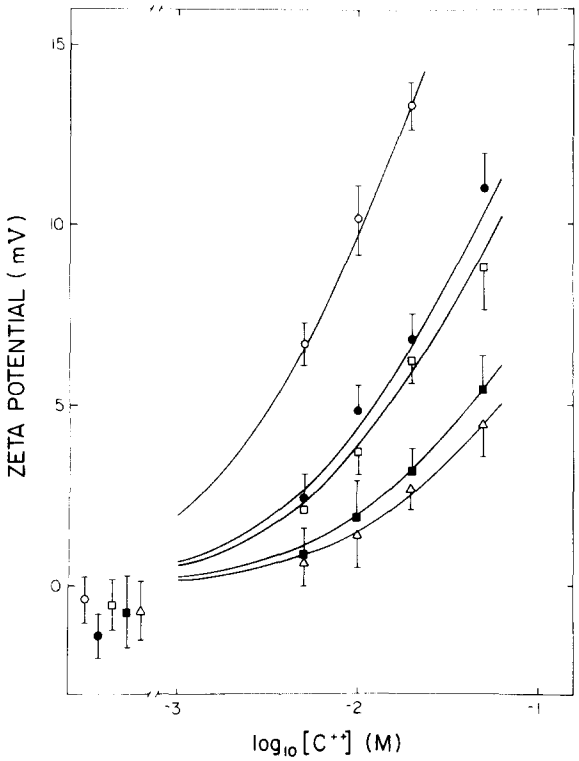

Fig. 1. The effects of the divalent cations Mn^{2+} (○), Mg^{2+} (●), Ni^{2+} (□), Sr^{2+} (■), and Ba^{2+} (△) on the zeta potentials of phosphatidylcholine vesicles. The curves through the points were drawn according to the combination of Eqns. 1—3, assuming that the area occupied by a lipid is 60 $Å^2$ and that the 1 : 1 dissociation constants are, respectively, 0.3, 1.0, 1.2, 2.8, and 3.6 M. If two phosphatidycholine molecules combine with one divalent cation the dissociation constants are half these values. The aqueous solutions contained 0.1 M NaCl buffered to pH 7.5 with 0.01 M Tris and the temperature was 25° C.

and a decrease in the conductance of a cation selective membrane (nonactin). To a crude first approximation these changes in conductance are equal in magnitude and opposite in direction. We average the magnitudes of the changes and calculate the change in the electrostatic potential in the interior of the membrane $\Delta\psi_{o-}$ by means of the formula:

$$G = G_{init} \exp(-zF\Delta\psi_{o-}/RT) \tag{13}$$

where G_{init} is the conductance measured in the presence of the carrier before the addition of calcium and G is the conductance measured after the addition of the indicated concentration of calcium to the aqueous solutions on both side of the planar black lipid membrane. RT and F have their usual significance and z is the valence of the charged permeant species (+1 for the potassium complex formed with nonactin [24], −1 for the HA_2^- complex formed between the neutral HA and anionic A^- species of DTFB [33]). The change in potential calculated from Eqn. 13 is indicated in the last row of Table I. The excellent agreement (5%) between the change in potential in the interior of the membrane estimated by the probes and the zeta potential (Fig. 2) is probably fortuitous. The results do indicate, however, that the adsorption of calcium pro-

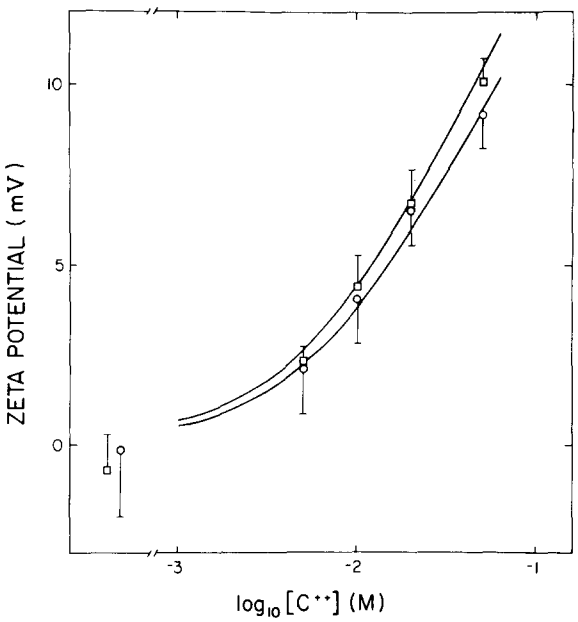

Fig. 2. The effects of the divalent cations Ca^{2+} (□) and Co^{2+} (○) on the zeta potentials of phosphatidylcholine vesicles. The curves through the points were drawn according to the combination of Eqns. 1—3, assuming that the area occupied by a lipid is 60 Å^2 and that the 1 : 1 dissociation constants for Ca^{2+} and Co^{2+} are 1.0 and 1.2 M. The aqueous solutions contained 0.1 M NaCl buffered to pH 7.5 with 0.01 M Tris and the temperature was 25° C.

duces no large change in the dipole or boundary potential [12] and that the measured values of the zeta potential should be a reasonable approximation to the value of the surface potential, ψ, which appears in Eqns. 2 and 3.

Fig. 3. illustrates how the percentage of bound phosphate groups, as determined from ^{31}P NMR measurements, varies as the concentration of cobalt in the bathing solution is increased. Note that there is a marked deviation from a linear isotherm, which is indicated in Fig. 3 by the dashed line. It is possible to describe the data with a normal Langmuir adsorption isotherm if one assumes that less than 10% of the phosphatidylcholine molecules are available to complex with cobalt. Such an assumption has been made to account for similar curves obtained with the lanthanides [34]. We prefer to fit the data with the combination of Eqns. 1, 2, 3 and 9, a combination we refer to as a Stern equation. A best fit was obtained with $k = 1/0.75$ M. The value of K was taken to be $(1/1.2\ M) \cdot (1/60\ Å^2)$, the value which gave a best fit to the zeta potential data.

TABLE I

THE EFFECT OF CALCIUM ON THE ELECTROSTATIC POTENTIAL IN THE INTERIOR OF BLACK LIPID MEMBRANES FORMED FROM PHOSPHATIDYLCHOLINE AS ESTIMATED BY "PROBE" MEASUREMENTS

	$[Ca^{2+}] = 24.4$ mM	$[Ca^{2+}] = 47.6$ mM
$\log_{10}(G/G_{init})$ for the negative probe DTFB	0.14 ± 0.04	0.20 ± 0.02
$\log_{10}(G/G_{init})$ for the positive probe nonactin	—0.12 ± 0.01	—0.16 ± 0.01
$\Delta\psi_o$ - (from Eqn. 6)	7.6 mV	10.5 mV

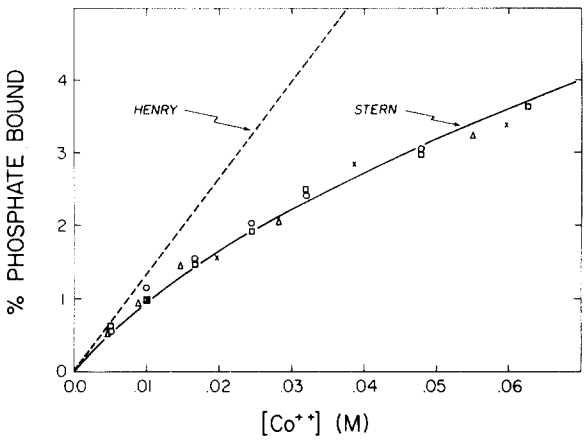

Fig. 3. The fraction of phosphatidylcholine molecules bound to cobalt as a function of cobalt concentration. The fraction of lipid phosphate groups bound to cobalt was calculated from the ^{31}P NMR linewidth (see text). The solid curve is the best fit to Eqns. 1, 2, 3 and 9 (see text). The dashed curve is the linear binding isotherm expected in the absence of surface potential effects. The experimental conditions were: sodium chloride, 0.095 M; egg phosphatidylcholine, 17 mg/ml; deuterium oxide, 20%; Tris chloride, 0.001 M, pH 7.5. $T = 20^{\circ}C$. The different symbols refer to separate experiments. Each sample was sonicated in the presence of the specified cobalt concentration.

Note that the combination of these equations can indeed describe the data in a satisfactory manner.

Table II illustrates the effect of an increase in the concentration of sodium chloride on the fraction of phosphate groups bound to cobalt. The observed results may be compared with the theoretical predictions obtained from the combination of Eqns. 1, 2, 3, and 9. At a given cobalt concentration an increase in the concentration of sodium chloride from 0.1 to 1.0 M should increase the fraction of bound phosphate groups. The observed enhancement in the binding, however, is somewhat larger than predicted theoretically. Grasdalen et al. [14] observed a similar discrepancy in their study of the adsorption of calcium to phosphatidylcholine. They chose to explain this discrepancy by invoking an adsorption of chloride ions to the membrane. While we cannot rule out this possibility, we know of no direct evidence that chloride ions do adsorb to phosphatidylcholine membranes *. There are a number of other possible explanations for this discrepancy between theory and experiment. For example, the increase in the monovalent salt concentration would be expected to enhance the discrete charge effect [35—37], and might have an effect on the membrane structure [38].

The hydrocarbon chains of all the phosphatidylcholines used in this study contained either double bonds or branched chains. Zeta potential measurements on saturated phosphatidylcholines (e.g. dimyristoyl phosphatidylcholine) indicate that the dissocation constant of divalent cations such as calcium to these membranes is about an order of magnitude lower than the values reported here. This is true both above and below the phase transition temperature of the saturated lipid.

* See footnote on page 343.

TABLE II

EFFECT OF NaCl ON THE FRACTION OF PHOSPHATE GROUPS OF PHOSPHATIDYLCHOLINE BOUND TO COBALT

All theoretical values were calculated assuming $T = 20°C$, $K = (1/1.2\ M) \cdot (1/60\ Å^2)$ and $k = 1/075\ M$, values chosen to provide a best fit to the zeta potential and ^{31}P NMR experiments conducted with [NaCl] = 0.1 M. With the exception that [NaCl] = 1 M, the conditions for the results listed in the last column were identical to those given in Fig. 3.

$[Co^{2+}]$ (mM)	[NaCl] = 0.1 M theoretical (%)	[NaCl] = 1 M theoretical (%)	[NaCl] = 1 M experimental (%)
13.2	1.2	1.5	1.7
22.8	1.8	2.4	2.9
32.2	2.3	3.1	3.8
44.6	2.9	3.9	4.4
56.6	3.4	4.6	5.6

Discussion

Our major conclusion is that the combination of the Grahame, Boltzmann and Henry equations can adequately describe both the zeta potential and ^{31}P NMR data when cobalt adsorbs to membranes formed from the zwitterionic lipid phosphatidylcholine. We anticipated that discrete charge effects might be more important for the adsorption of divalent than monovalent ions [35—37], but if this effect were of overriding importance, we would have obtained steeper potential vs. concentration curves than we actually observed in Figs. 1 and 2, and the deviation from Henry's law in Fig. 3 would have been less marked than observed. Our conclusion that the data are well described by Eqns. 1—3 must, however, be tempered by the caveat that we do not know the stoichiometry of the binding between the phosphatidylcholine lipids and cobalt. This allowed us to chose the ratio of 1 : 1 and 2 : 1 complexes to fit the ^{31}P NMR data.

The analysis of the zeta potential data involves the number of adsorbed cobalt ions per unit area *, while the analysis of the ^{31}P NMR data involves the number of phosphate groups bound to cobalt **. To relate the two sets of data we need to assume a stoichiometry for the phosphatidylcholine · cobalt com-

* The Grahame equation, which relates the zeta or surface potential to the number of adsorbed cobalt ions, should be tested by independent experiments for the case where divalent ions such as calcium and cobalt adsorb. It is difficult, however, to see how Eqn. 3 can be tested directly (for example, by equilibrium dialysis or Hummel and Dryer type experiments) for these ions because they adsorb so weakly to phosphatidylcholine membranes.

** The accuracy of the ^{31}P NMR determination of f depends on the accuracy of the assumption that $\Delta\omega_M$ is identical for phosphatidyl choline in bilayer membranes and glycerophosphoryl choline in solution [17]. The other major assumptions involved in determining the stoichiometry are that the dissociation constants and area per lipid molecule are identical for the unsonicated vesicles and both surfaces of the sonicated vesicles, assumptions which are probably incorrect. For example, we assumed throughout our analysis that the lipid area was 60 $Å^2$, an appripriate assumption for black lipid membranes [29]. X-ray studies indicate, however, that the area per egg phosphatidylcholine molecule in multilamellar dispersions is probably between 70 and 75 $Å^2$ [39,40] while Huang and Mason [41] have recently estimated that the average area per lipid head group in a sonicated vesicle is 70 $Å^2$. While it is difficult to accurately estimate the consequences of the effects discussed in footnotes * and **, the possible errors involved could significantly alter the calculated distribution between 1 : 1 and 2 : 1 complexes. We also ignored activity coefficient effects in this paper, although they could readily be incorporated into the binding formalism if one assumes that the activity coefficient of the adsorbing cation in the aqueous phase at the membrane solution interface is equal to the activity coefficient of the ion in the bulk aqueous solution [14].

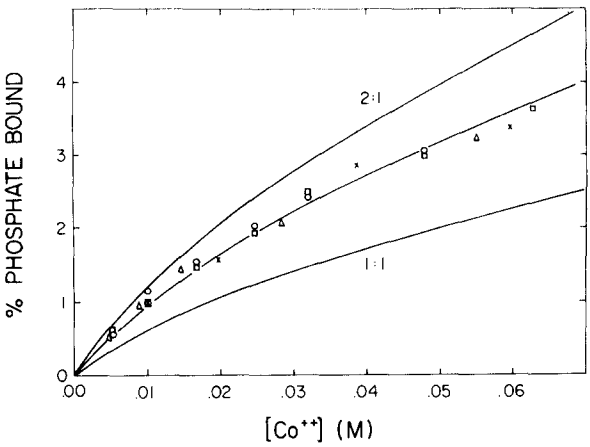

Fig. 4. The fraction of phosphatidylcholine molecules bound to cobalt as a function of cobalt concentration. The fraction of lipid phosphate groups bound to cobalt was calculated from the ^{31}P NMR linewidth (see text). The solid curves at the top and the bottom of the figure are calculated from Eqns. 1, 2, 3 and 9 using the value of K derived from the zeta potential data and assuming either a 1 : 1 or a 2 : 1 stoichiometry. The solid curve through the data points is the best-fit to Eqns. 1, 2, 3 and 9 assuming that both 1 : 1 and 2 : 1 complexes are simultaneously present (see text). Experimental details are identical to those given for Fig. 3.

plex. The upper and lower curves in Fig. 4 illustrate the theoretically predicted curves for the ^{31}P NMR data assuming either a 2 : 1 or a 1 : 1 stoichiometry, and using the value of K derived from the fit of the Stern equation to the zeta potential data. The ^{31}P NMR data lie between the theoretically predicted curves for 2 : 1 and 1 : 1 stoichiometry. It is only to the extent that the data lie between the two curves that we can conclude the Stern equation provides an adequate description of experimental reality. The data can be quantitatively fit by assuming that both 2 : 1 and 1 : 1 complexes are present simultaneously. From the theoretical fit to the data (middle line in Fig. 4) it is calculated that one lipid would be involved in a 1 : 1 complex for every three lipids involved in 2 : 1 complexes with cobalt *. Assuming that the surface densities of binding sites are $1/b$ and $1/2b$, the dissociation constants for the 1 : 1 and the 2 : 1 complexes are calculated to be K_{D1} = 3.0 M and K_{D2} = 1.0 M, respectively.

The ^{31}P NMR results allow us to draw some conclusions about the lifetime and molecular nature of the phosphatidylcholine · cobalt complex (Appendix). The calculated value of the ^{31}P NMR shift for the phosphatidylcholine · cobalt complex, $\Delta\omega_M$, is very similar to the value measured for the $Co_3(PO_4)_2$ crystal [42,16]. This implies that the phosphate group of phosphatidylcholine is incorporated into the first co-ordination sphere of the cobalt ion. The lifetime of phosphatidylcholine in the complex, τ_M, is $3 \cdot 10^{-6}$ s at 20°C and the lifetime of cobalt on the membrane should be roughly equal to this value. A knowledge of the dissociation constant and the lifetime allows us to calculate the effective rate constant for formation of the complex. This rate constant is at least two orders of magnitude smaller than the value calculated by assuming that the interaction of cobalt with phosphatidylcholine molecules in the membrane proceeds by a simple one-step diffusion-controlled reaction. This does not seem to

* See second footnote on page 349.

be a consequence of the membrane structure because the glycerophosphorylcholine complex has similar values for the dissociation constant and the lifetime of the cobalt complex [17].

Our zeta potential results obtained with calcium may be used to test whether the major conclusion we reached with respect to the adsorption of cobalt to phosphatidylcholine membranes is also applicable to the adsorption of calcium to phosphatidylcholine membranes. Hauser et al. [43] reported neither shifts nor broadening but both Grasdalen et al. [14] and Hutton et al. [15] observed shifts in the phosphate peak of the head group in the outer surface of the vesicles on addition of calcium. In Fig. 5 we fit their data to the combination of Eqns. 1—3, using for K the value deduced from the zeta potential measurements of Fig. 2. In contrast to the ^{31}P NMR data obtained with cobalt, there is no calibration factor available to interpret the experimentally observed shift in the ^{31}P NMR data obtained with calcium in terms of the fraction of bound phosphate groups. The curve was arbitrarily scaled to the 0.4 M point by assuming either 1 : 1 stoichiometry with a dissociation constant of 1.0 M, or by assuming a 2 : 1 stoichiometry with a dissociation constant of 0.5 M. The fit to the data is satisfactory which confirms the conclusion of Grasdalen et al. [14] that their results are consistent with the Stern equation.

Hauser et al. [30] observed the binding of Ca^{2+} to phosphatidylcholine membranes by measuring its effect on the binding of Eu^{3+}, using NMR to monitor the chemical shift in the proton $N(CH_3)_3$ signal of phosphatidylcholine. The procedure to be used for calculating a binding constant when a divalent cation competes with a trivalent cation is, however, unclear.

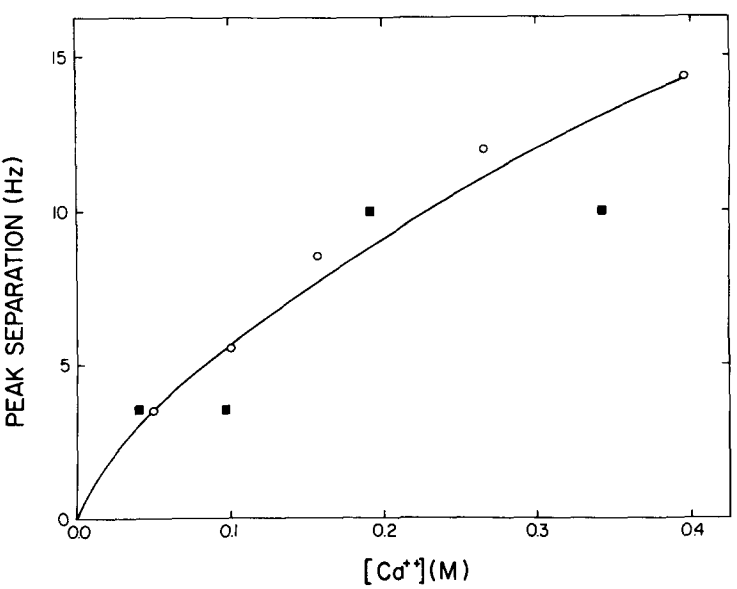

Fig. 5. The shift of the ^{31}P NMR signal from sonicated phosphatidylcholine vesicles on the addition of calcium chloride. The ^{31}P NMR data are taken from Grasdalen et al. [14] (○) and Hutton et al. [15] (■). In order to fit the data to Eqns. 1, 2, 3 and 9 the theoretical curve was scaled so that it coincided with the experimental peak separation at 0.4 M calcium chloride. The value of the constant K was assumed to be identical to that derived from the zeta potential measurements given in Fig. 2 (see text).

Several groups [44—46] have reported apparent strong interactions between calcium and phosphatidylcholine membranes (dissociation constants on the order of 10^{-3} M) based on studies with the fluorescent probe 1-anilino-8-napthalenesulfonate (ANS). McLaughlin et al. [3] pointed out, however, that ANS adsorbs to membranes formed from zwitterionic lipids and, at the concentrations used by these investigators, itself produces a substantial negative surface potential, a conclusion we have confirmed by means of zeta potential measurements (unpublished). When calcium as added to these solutions it screens the negative charges, allowing more ANS to adsorb, and thus produces the observed increase in fluorescence without itself adsorbing significantly to the phosphatidylcholine membranes at low concentrations.

Other techniques are, in general, not sensitive enough to contribute much to our understanding of the rather weak interaction of calcium with phosphatidylcholine membranes. By studying the enhancement of the beta emission of ^{45}Ca in aqueous solution on spreading of a monolayer of phosphatidylcholine, a number of groups [43,47,48] concluded that there was essentially no interaction of this ion with phosphatidylcholine. The titration studies of Dervichian [49], where little change in pH was observed on addition of Ca^{2+} to phosphatidylcholine, are also consistent with our results. It should be possible to detect the adsorption of calcium and other divalent cations to phosphatidylcholine monolayers by means of direct surface potential measurements. A number of investigators [50—52] have reported significantly larger changes in surface potential than we observed with zeta potential measurements. Collacico [53], however, argued that these results were due to a negative contaminant in the phosphatidylcholine samples. In a later publication, Shah and Shulman [54] report smaller changes in surface potential on addition of calcium to dioleoyl or egg phosphatidylcholine monolayers, changes which agree with our zeta potential measurements within a factor of two.

There have been two studies of the binding of manganese ions to phosphatiylcholine vesicles. Puskin [55] concluded that the dissociation constant was 0.11 M, whereas Nolden and Ackermann [31] estimated, by a different technique, that the dissociation constant was about 0.02 M. The value we deduce from zeta potential measurements, also assuming 1 : 1 stoichiometry, is 0.3 M (Fig. 1), in qualitative agreement with Puskin [55]. The experiments of Nolden and Ackermann were done in pure water without buffer. A contamination of 0.5% (about the level of negatively charged contaminants we found to be introduced by our sonication procedure) would produce an enormous negative surface potential under their experimental conditions. They added phosphatidylcholine to a solution containing manganese at a concentration of $2.5 \cdot 10^{-5}$ M. The Grahame equation from the theory of the diffuse double layer predicts that the potential at the surface of these vesicles should be of the order of —40 mV. The local concentration of Mn^{2+} would thus be a factor of 20 higher than in the bulk aqueous solution and the dissociation constant they deduce would be also in error by this factor.

In summary, we know of no study of the adsorption of divalent ions to phosphatidylcholine membranes which disagrees with our conclusion that the Stern equation can adequately describe the association. In fact, this conclusion allows one to understand results which have appeared puzzling to other investi-

gators. The Stern equation predicts why an increase in the concentration of an 'indifferent' electrolyte like NaCl will increase the binding of a changed molecule to phosphatidylcholine membranes. If the substance which adsorbs is cationic (e.g. Ca^{2+} [14]; Co^{2+}, Table II; lanthanides [14,30,43]), the additional chloride ions will screen the adsorbed charges, lower the magnitude of the surface potential and allow more adsorption of the cation. A similar screening role is played by sodium ions if the substance which adsorbs is an anion [22,23]. It is not necessary to postulate that the effect is due to 'steric hindrance due to the extended conformation of the lecithin group' [43] or to invoke [30] the theory of Gillespie [56]. While Gillespie's theory does predict that NaCl should enhance the binding of calcium to phosphatidylcholine membranes, it is based on the assumption that the head group of phosphatidylcholine is in a fixed extended conformation, which now seems unlikely [57—59]. It also makes the erroneous predictions that calcium chloride and sodium chloride should significantly affect the surface potential of phosphatidylcholine monolayers at concentrations below 0.001 M.

Studies on the adsorption of divalent cations to membranes comprised of mixtures of neutral and negative lipids are in progress and should yield results which bear on the interesting ability of divalent ions to induce the phase separation [60,61] of lipids and the fusion [62—65] of membranes.

Appendix

Phosphorus nuclear magnetic resonance (^{31}P NMR) spectra are very sensitive to the binding of paramagnetic divalent cations. Since divalent cations are presumed to bind at the phosphate group of phosphatidylcholine membranes, ^{31}P NMR is an ideal technique to study the interaction of paramagnetic divalent cations with these membranes.

From a complete analysis of the effects of cobalt on the ^{31}P NMR spectrum of phosphatidylcholine vesicles we can obtain three types of information: (1) the fraction of phosphate groups in phospholipid · cobalt complexes, f; (2) the lifetime of the phospholipid · cobalt complex, τ_M; (3) the molecular nature of the phospholipid · cobalt complex (i.e., if it is an inner or outer-sphere complex).

In order to obtain this information the temperature and magnetic field dependence of the effects of cobalt on the ^{31}P NMR spectrum must be investigated. The complete equations describing these effects are rather cumbersome [66,67]. However, three approximations allow a considerable simplification of these equations. First, since only a small fraction of the phosphate groups are bound to cobalt at any one time (less than 1% in the experiments discussed in this Appendix) the observed ^{31}P NMR signal arises almost completely from the free phosphate groups. Second, as previously discussed [17], the frequency dependence of the observed linewidth in the high-temperature region allows us to deduce that, throughout the entire temperature range of the experiments, $1/T_{2M} << \Delta\omega_M{}^2\tau_M$ where $1/\pi T_{2M}$ is the width and $\Delta\omega_M$ is the shift of the ^{31}P NMR signal of the bound phosphate groups. Third, from the values of T_{1P} (0.175 s), the observed longitudinal relaxation time and $\Delta\omega_P$ (11 780 rad/s, the observed shift at 145 MHz (4 mM cobalt, 77°C), it is calculated that in the high

temperature region of the 145 MHz data $(\Delta\omega_M)/(1/T_{1M}) = 2061$ where T_{1M} is the longitudinal relaxation time of the bound phosphate groups. Since $T_{1M} \approx T_{2M}$, it may be concluded that:

$$(\Delta\omega_M)/(1/T_{2M}) >> 1$$

for the data shown in Fig. 6.

With these three approximations, the complete equations for the effects of cobalt on the observed linewidth, $1/\pi T_{2P}$, and the observed shift, $\Delta\omega_P$, simplify to [17]:

$$1/T_{2P} = f \frac{\Delta\omega_M^2 \tau_M}{1 + \Delta\omega_M^2 \tau_M^2} \tag{A1}$$

and

$$\Delta\omega_P = f \frac{\Delta\omega_M}{1 + \Delta\omega_M^2 \tau_M^2} \tag{A2}$$

As previously discussed [17], the temperature dependence of f and τ_M is assumed to be of the form: $\tau_M = \tau_M^\circ \exp(\Delta H_1/RT)$; $f = f^\circ \exp(\Delta H_2/RT)$ and $\Delta\omega_M$ is assumed to be inversely proportional to temperature and directly proportional to the magnetic field strength. Once $\Delta\omega_M$ has been determined, the temperature and magnetic field strength dependence of $1/T_{2P}$ and $\Delta\omega_P$ allow us to determine both τ_M° and f°. The value of $\Delta\omega_M$ is assumed to be the same as the value determined for glycerophosphorylcholine, −1442 ppm [17]. Eqns. A1 and A2 may then be used to theoretically fit the data shown in Fig. 6 (solid lines). The values of τ_M° and f° derived from this fit are shown in Table III.

There are two possible complications that might arise in the analysis of the effects of cobalt on the sonicated phosphatidylcholine vesicles. First, the aniso-

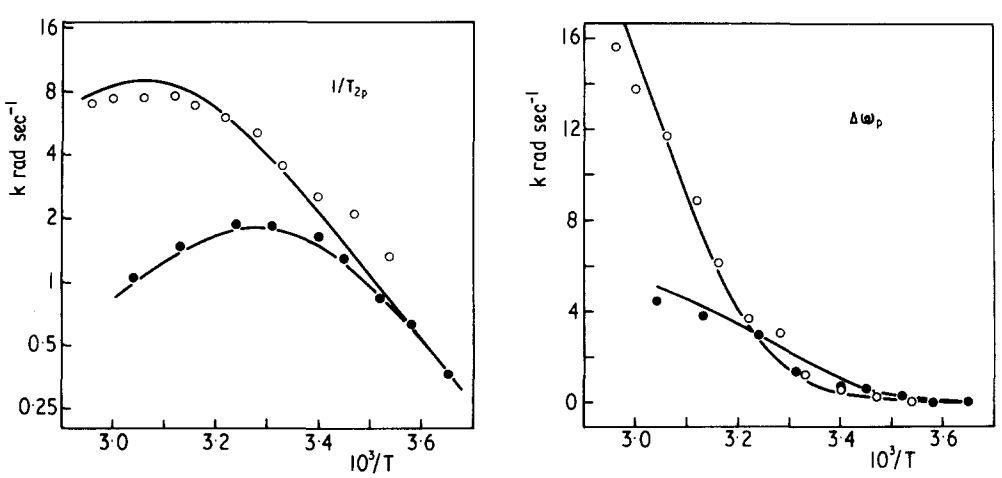

Fig. 6. The observed linewidth, $1/T_{2P}$, and the line-shift, $\Delta\omega_P$, for sonicated phosphatidylcholine vesicles in the presence of cobalt. The data were taken at two different frequencies (●, 36.4 MHz; ○, 129 MHz) as a function of temperature. The experimental conditions were: phosphatidylcholine, 30 mg/ml; sodium chloride, 0.1 M; Tris chloride, 0.01 M, pH 7.5. The solid curves were calculated from the simultaneous best-fit of all the data to Eqns. A1 and A2 (see Appendix).

TABLE III

CALCULATED PARAMETERS FOR THE COBALT · PHOSPHATIDYLCHOLINE COMPLEX

Parameters are defined in the text.

τ_M°	$0.5 \cdot 10^{-13}$ s
ΔH_1	10.5 kcal/mol
f°	2.4 *
ΔH_2	−3.5 kcal/mol
$\Delta\psi_M$	−1442 ppm (70°C) **

* Calculated for 0.006 M cobalt chloride and the conditions given in Fig. 6.

** Measured value for the glycerophosphorylcholine complex [17] (see Discussion).

tropy in the cobalt-phosphate interaction might not be averaged by the relatively long Brownian rotational time of the vesicles (about $2 \cdot 10^{-6}$ s at 20°C). Thi This effect is difficult to quantitatively rule out without a complete resolution of the interaction into anisotropic and isotropic components, a resolution which is not available at the present time. However, the much shorter Brownian rotational time for the glycerophosphorylcholine · cobalt complex will completely average any anisotropy in the cobalt-phosphate interaction. The fact that the shape of the linewidth versus $1/T$ curves for phosphatidylcholine membranes may be superimposed on the linewidth versus $1/T$ curves for glycerophosphorylcholine [17] implies that the anisotropy is also averaged in the membranes. Second, intermolecular (pseudocontact) cobalt-phosphate interactions might affect the ^{31}P NMR spectrum of phospholipids which were not directly bound to cobalt. A simple estimate of the intermolecular pseudocontact interaction shows that it would be less than 10% of the intramolecular interaction. Since the relevant correlation time for the intermolecular interaction would be the lateral diffusion time of the lipids (about $2 \cdot 10^{-7}$ s at 20°C) the intermolecular effects will be in fast-exchange throughout the entire tempetemperature range. Since the observed shifts approach zero at the low temperatures, the intermolecular effects can be shown to be negligible.

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