Adsorption of Divalent Cations to Bilayer Membranes Containing Phosphatidylserine

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ABSTRACT The Stern equation, a combination of the Langmuir adsorption isotherm, the Boltzmann relation, and the Grahame equation from the theory of the diffuse double layer, provides a simple theoretical framework for describing the adsorption of charged molecules to surfaces. The ability of this equation to describe the adsorption of divalent cations to membranes containing brain phosphatidylserine (PS) was tested in the following manner. Charge reversal measurements were first made to determine the intrinsic 1:1 association constants of the divalent cations with the anionic PS molecules: when the net charge of a PS vesicle is zero one-half of the available sites are occupied by divalent cations. The intrinsic association constant, therefore, is equal to the reciprocal of the divalent cation concentration at which the mobility of a PS vesicle reverses sign. The Stern equation with this association constant is capable of accurately describing both the zeta potential data obtained with PS vesicles at other concentrations of the divalent cations and the data obtained with vesicles formed from mixtures of PS and zwitterionic phospholipids. Independent measurements of the number of ions adsorbed to sonicated PS vesicles were made with a calcium-sensitive electrode. The results agreed with the zeta potential results obtained with multilamellar vesicles. When membranes are formed at 20°C in 0.1 M NaCl, the intrinisc 1:1 association constants of Ni, Co, Mn, Ba, Sr, Ca, and Mg with PS are 40, 28, 25, 20, 14, 12, and 8 M^{-1}, respectively.

INTRODUCTION

Although calcium exerts many interesting effects on lipid bilayer membranes containing phosphatidylserine (PS), there is no satisfactory theory that explains its ability to induce either phase transitions and separations of the lipid components or aggregation and fusion of the membranes (e.g., Träuble and Eibl, 1974; Jacobson and Papahadjopoulos, 1975; MacDonand et al., 1976; Forsyth et al., 1977; Papahadjopoulos et al., 1977; Van Dijck et al., 1978; Portis et al., 1979; Ohki and Duzgunes, 1979; Zimmerberg et al., 1980; Cohen et al., 1980). A quantitative description of the adsorption of Ca to PS is necessary, in our opinion, to understand these phenomena. Information about

the association of Ca with PS also has direct physiological significance. For example, the process of visual transduction may involve the diffusion of Ca within the cytoplasm of a retinal rod cell. It is not widely recognized that the effective diffusion coefficient of Ca in the cytoplasm must be 1–2 orders of magnitude lower than the diffusion coefficient in water because binding of Ca to PS in the intracellular disk membranes will occur concomitantly with diffusion (McLaughlin and Brown, footnote 1). Ca and other cations can also affect the electrostatic potential adjacent to ion-selective channels in excitable membranes (e.g., Gilbert and Ehrenstein, 1969; Begenisich, 1975; Hille et al., 1975; Schauf, 1975). The source of the negative charges adjacent to these channels is unknown but it could be PS, the major charged lipid in the bilayer component of the membrane. A comparison of the relative ability of the divalent cations to bind to PS and to shift the conductance-voltage curves of an excitable membrane would help to test this possibility.

The apparent association constant, K_A, of the Ca-PS complex depends on the electrostatic potential, ψ_o, in the aqueous phase adjacent to the surface of the membrane. This potential causes calcium ions to be concentrated at the surface of the membrane according to the Boltzmann expression. The "intrinsic" association constant, K_I, (e.g., Rice and Nagasawa, 1961) takes this concentrating effect into account and should be independent of the surface potential. By definition,

$$K_I = K_A \exp\left(+zF\psi_o/RT\right), \tag{1}$$

where z is the valence of the adsorbing ion, F is the Faraday, R is the gas constant, and T is the absolute temperature.[2] The published values of K_I for the Ca-PS complex vary over some five orders of magnitude, from 10^4 to 10^{-1} M^{+1}. This is clearly an unacceptable range.

In this paper we report new estimates, based on microelectrophoresis measurements, of the effect of Ca on the zeta potential of membranes containing PS and new estimates, based on measurements with a calcium sensitive electrode, of the number of calcium ions bound to PS membranes. When the membranes are formed from PS or from a mixture of PS and a zwitterionic lipid such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE), all the data are consistent with the simple Gouy-Chapman-Stern model, assuming that calcium forms mainly 1:1 complexes with PS and that the intrinsic association constant, K_I, is ~ 10 M^{-1}. We argue briefly below, and in more detail in the Discussion, that the data available in the literature are also consistent with this conclusion.

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[1] McLaughlin, S., and J. Brown. The diffusion of calcium in the cytoplasm of retinal rod outer segments. *J. Gen. Physiol.* **77**:475–487.

[2] The potential in Eq. 1 is, strictly speaking, the "micropotential" or potential at the binding site. In this report we ignore any "discreteness of charge" effects (e.g., Grahame, 1958; Levine, 1971; Cole, 1969; Brown, 1974; Nelson and McQuarrie, 1975; Tsien, 1978; Wang and Bruner, 1978) and assume that ψ_o can be approximated by the average potential in the aqueous phase at the membrane-solution interface. ^{31}P NMR experiments that utilize the number of bound cobalt ions as a probe of the potential at the binding site support this assumption (unpublished results).

The low values of K_I in the literature were obtained from estimates of the effect of Ca on the electrostatic potential either within a planar PS bilayer (McLaughlin et al., 1971; $K_I = 0.1$ M^{-1}) or above a PS monolayer (Ohki and Sauve, 1978; $K_I = 0.1$ M^{-1}). In these studies only the changes in electrostatic potential produced by calcium could be measured, and it was assumed that the alkali metal cations in the solution do not adsorb specifically to PS but exert only a "screening" effect, as predicted by the Gouy equation from the theory of the diffuse double layer. There is now reasonably good circumstantial evidence that the alkali metal cations do adsorb to PS (Abramson et al., 1961; Hauser et al., 1975; Puskin, 1977; Nir et al., 1978; Kurlnd et al., 1979; Eisenberg et al., 1979) and that this adsorption can be described by the Stern equation, a combination of the Gouy, Boltzmann, and Langmuir expressions (Eisenberg et al., 1979). Thus, the magnitude of the electrostatic potential adjacent to a PS membrane formed in a solution of alkali metal cations is lower than that predicted by the Gouy equation, which accounts only for the screening effect of the monovalent cation. It follows that the value of the calcium intrinic association constant required to explain surface potential data obtained in the presence of this divalent cation is higher than the 0.1 M^{-1} value previously used. In the Discussion we demonstrate that the surface potential data in the literature are consistent with a $K_I \simeq 10$ M^{-1} for the Ca-PS complex.

A high value of the intrinsic association constant ($K_I \simeq 10^4$ M^{-1}) was obtained from measurements of the uptake of radioctive calcium under a monolayer formed from PS (Hauser et al., 1976). As Ohki and Sauve (1978) pointed out, this technique does not distinguish between the radioactive calcium ions chemically bound to the surface of the monolayer and the ions sequestered in the double layer a few ångströms from the surface. Only when the counterions in the diffuse double layer consist mainly of cations other than calcium is it possible to interpret the excess [45]Ca radioactivity measured in these experiments in terms of the number of calcium ions specifically bound to PS. Some of the experiments of Hauser et al. (1976) were conducted under these conditions. For example, Hauser et al. (1976) have measured the value of K_A to be 2×10^5 M^{-1} in the presence of 3 mM NaCl and 0.1 μM Ca. It is easy to show, within the context of the Gouy-Chapman-Stern theory discussed below, that most of the counterions in the diffuse double layer are sodium rather than calcium ions under these conditions. The surface potential, which may be approximated by the zeta potential when the concentration of salt is low and the Debye length is large (Eisenberg et al., 1979), is about −150 mV, and the intrinsic association constant is thus of the order 10 M^{-1}, in agreement with the results reported here.

There are many techniques available for studying the interaction of calcium with lipids in bilayer membranes, and there are advantages and disadvantages to each of the approaches. In particular, there are four major advantages to the electrophoresis approach adopted here. First, the lipids in the large (1–20 μm) multilamellar vesicles used in this study are not in a strained configuration, in contrast to the lipids in small sonicated vesicles. Second, the bilayers do not contain any organic solvents, as do planar black lipid membranes

formed by the conventional Mueller-Rudin method. Third, electrophoresis measurements are made on individual multilamellar vesicles, in contrast to electrode or dialysis measurements, which are made on an ensemble of sonicated vesicles. This allows measurements to be made at calcium concentrations that aggregate and precipitate most of the vesicles. Fourth, the electrophoresis approach provides an absolute measure of the zero potential. When sufficient calcium is added to the aqueous solution to reverse the mobility of the PS vesicles, the net charge and surface potential are both zero. Capacitance (Schoch et al., 1979) and conductance (McLaughlin et al., 1971; Muller and Finkelstein, 1972; Cohen and Moronne, footnote 3) measurements on bilayer membranes and direct surface potential measurements on PS monolayers (Bangham and Papahadjopoulos, 1966; Papahadjopoulos, 1968; Ohki and Sauve, 1978) determine only the *change* in the surface potential. The reversal of charge technique, which has long been used in colloid and interface chemistry, can be employed to "calibrate" the other techniques (Eisenberg et al., 1979). The major disadvantage of the electrophoresis measurements is that the potential is determined at an idealized plane of shear rather than at the surface of the membrane.

THEORY

The zeta potential, ζ, the electrostatic potential at the hydrodynamic plane of shear, is calculated from the measured value of the electrophoretic mobility, u, by the Helmholtz-Smoluchowski equation:

$$\zeta = u\eta/\epsilon_r\epsilon_o, \tag{2}$$

where ϵ_r is the dielectric constant of the aqueous phase, η is the viscosity of the aqueous phase and ϵ_o is the permittivity of free space. Wiersema et al. (1966) or Overbeek and Wiersema (1967) may be consulted for a derivation of this equation. In a previous publication we showed that when phospholipid vesicles are formed in a solution of the alkali metal cations, the observed zeta potentials are consistent with the assumptions that: (*a*) the plane of shear is 2 Å from the surface of the membrane and (*b*) the profile of the potential in the aqueous phase may be described by the classic theory of the diffuse double layer (Eisenberg et al., 1979). We make the same two assumptions in our analysis of the experimental data reported here. The appropriate equations[4] for the potential profile when the aqueous solution contains both monovalent and divalent cations have been derived by Abraham-Schrauner (1975).

The relationship between the surface charge density, σ, and the surface

[3] Cohen, J., and M. Moronne. 1980. Personal communication.

[4] See Eqs. 1 and 7 of Abraham-Schrauner (1975). When the membrane bears a positive charge, the exponent +2 of the hyperbolic tangent function of her Eq. 1 becomes a −2 exponent (i.e., $\tanh^2$ becomes $\coth^2$), as noted by Abraham-Schrauner (1977), and the inverse hyperbolic tangent function (archtanh or $\tanh^{-1}$) in her Eq. 7 becomes an inverse hyperbolic cotangent function (archcoth or $\coth^{-1}$).

potential, ψ_o, is given by the Grahame (1947) equation from the Gouy-Chapman theory of the diffuse double layer:

$$\sigma = \pm \{2\epsilon_r\epsilon_o RT \Sigma_i C_i [\exp(-z_i F\psi_o/RT) - 1]\}^{1/2}, \tag{3}$$

where C_i is the concentration of ions of valence z_i in the bulk aqueous phase.

The concentrations of monovalent and divalent cations in the aqueous phase at the surface of the membrane [$C^+(o)$ and $C^{++}(o)$, respectively] are assumed to be related to their bulk aqueous concentrations (C^+ and C^{++}, respectively) by the Boltzmann equation:

$$C^+(o) = C^+\exp(-F\psi_o/RT) \tag{4}$$
$$C^{++}(o) = C^{++}\exp(-2F\psi_o/RT).$$

The monovalent cation is assumed to bind to the negative lipid, P^-, and form 1:1 complexes, C^+P^-. We assume that the Langmuir adsorption isotherm is valid (Eisenberg et al., 1979), which is equivalent to writing

$$\{C^+P^-\} = K_1\{P^-\}C^+(o), \tag{5}$$

where K_1 is an intrinsic association constant (M^{-1}) and the braces denote a surface concentration (i.e., the number of molecules on the surface of the membrane per unit area).

The divalent cation is also assumed to form only 1:1 complexes, $C^{++}P^-$, with the negative lipid (see below), and we again assume that the Langmuir adsorption isotherm is valid:

$$\{C^{++}P^-\} = K_2\{P^-\}C^{++}(o), \tag{6}$$

where K_2 is an intrinsic association constant. When zwitterionic lipids, P, are present in the membrane, the divalent cation is assumed to form 1:1 complexes with these lipids:

$$\{C^{++}P\} = K_3\{P\}C^{++}(o), \tag{7}$$

where K_3 is an intrinsic association constant.

The total surface concentration of the negative lipid, $\{P^-\}^{tot}$, is the sum of the free and bound surface concentrations:

$$\{P^-\}^{tot} = \{P^-\} + \{C^+P^-\} + \{C^{++}P^-\}. \tag{8}$$

The total surface concentration of the zwitterionic lipid, $\{P\}^{tot}$, is the sum of the free and bound surface concentrations:

$$\{P\}^{tot} = \{P\} + \{C^{++}P\}. \tag{9}$$

Algebraic manipulation of Eqs. 5-9 yields:

$$\sigma = \frac{-\{P^-\}^{tot}[1 - K_2 C^{++}(o)]}{[1 + K_1 C^+(o) + K_2 C^{++}(o)]} + \frac{2K_3 \{P\}^{tot} C^{++}(o)}{[1 + K_3 C^{++}(o)]}. \tag{10}$$

The combination of Eqs. 3, 4, and 10 is referred to as a Stern[5] equation. The equation is solved numerically for the surface potential, ψ_o, as a function of the concentration of divalent cations in the bulk aqueous phase. The theoretical value of the zeta potential, the potential at a distance 2 Å from the surface of the membrane, is calculated from ψ_o by means of diffuse double layer theory (Abraham-Schrauner, 1977). We assume that each negative and zwitterionic phospholipid occupies an area of 70 $Å^2$ in the membrane. We also assume that phase separation does not occur under our experimental conditions when bilayer membranes containing both negative and zwitterionic lipids are exposed to divalent cations (Papahadjopoulos et al., 1974 and 1977).

MATERIALS AND METHODS

Similar results were obtained with salts of ultrapure (Ventron Corp., Danvers, Mass.; Spex Industries, Inc., Metuchen, N. J.) an analytical (Fisher Scientific Co., Fairlawn, N. J.) quality. Water was purified with a Suyper -Q system (Millipore Corp., Bedford, Mass.). The concentrations of electroyltes in the stock solutions were checked by measuring the conductivity at 20°C. All phospholipids (bovine brain phosphatidylserine, dimyristoylphosphatidylserine, dimyristoylphosphatidylglycerol, egg phosphatidylcholine, bacterial phosphatidylethanolamine, egg phosphatidylethanolamine, and phosphatidylglycerol transphosphatidylated from egg phosphatidylcholine) were obtained from Dr. W. Shaw of Avanti Biochemicals Inc. (Birmingham, Ala.), who prepared the negative lipids by treatment with ethylenediaminetetraacetate (EDTA) to remove divalent ion contaminants. Blycerolmonooleate (monoolein) was obtained from Nu-chek-prep (Elysian, Minn.). The purity of the lipids was established by one-dimensional thin-layer chromatography in chloroform-methanol-water (65:25:4); 0.5 mg of each lipid chromotraphed as a single spot.

Multilamellar vesicles for the electrophoresis experiments were formed by the method of Bangham et al. (1974). When forming vesicles from mixtures of phosphatidylserine and glycerolmonooleate we noted that reproducible results could only be obtained if the dried lipid mixture was resuspended in chloroform and dried a second time. Electrokinetic mobilities were measured with Rank Bros. Mark I microelectrophoresis machines (Bottisham, Cambridge, U. K.). The machines were focused at the stationary layer (Henry, 1938), and the current was monitored to ensure that significant electrode polarization did not occur. Control experiments revealed that the mobilities of the vesicles in the presence of Ca did not change significantly over a time-course of several hours. Aggregation occurred with vesicles formed from some lipids and lipid mixtures upon addition of divalent cations. All measurements reported in the figures were made on the remaining unaggregated multilamellar vesicles, although no significant difference was observed between the mobility of the vesicles and the mobility of the aggregates. Control experiments were performed with extremely dilute solutions of multilamellar vesicles, where aggregation proceeded very slowly, to further ensure that the unaggregated vesicles did not constitute an anomalous subpopulation.

Unilamellar vesicles for the electrode experiments were formed by drying a chloroform solution of lipids under high vacuum for a minimum of 4 h, resuspending the

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[5] Davies and Rideal (1963, p. 85) or Sparnaay (1972, p. 104) may be consulted for a discussion of the minor difference between the combination of the Langmuir, Boltzmann, and double layer equation used here and Stern's original formulation.

lipids in the appropriate aqueous solution, and sonicating with a Branson Sonic Power Co. W185 sonifier (Danbury, Conn.) under a nitrogen atmosphere. The sonicated material was centrifuged at 120,000 g for 45 min at 30°C. The upper two-thirds of the supernate was utilized for the electrode measurements (Barenholz et al. 1977). The concentration of phospholipid in the solution was determined by phosphate analysis (Lowry and Tinsley, 1974). Measurements of the free concentrations of calcium in the solution were made in a temperature-controlled Teflon (E. I. de Nemours & Co., Inc., Wilmington, Del.) cup with calcium-sensitive electrodes from Nuestra Research Inc. (Salt Lake City, Utah). All results reported were obtained with electrodes for which the calcium response was Nernstian in the concentration range under consideration (5–500 μM) and for which the calibration readings before and after the experiment agreed within ±1 mV.

RESULTS

PS Membranes

Fig. 1 illustrates the effect of divalent cations on the zeta potentials of multilamellar vesicles formed from PS in 0.1 M NaCl.[6] The addition of divalent cations to the solution bathing the vesicles causes the zeta potential to become less negative and, at sufficiently high concentrations, to pass through zero and become positive. (This is also true for PS vesicles on addition of 0.2 M MgCl$_2$; data not shown). The dashed line in Fig. 1 B illustrates the theoretical prediction of the Stern equation if it is assumed that the divalent cations do not adsorb to PS (i.e., $K_1 = 0.6$; $K_2 = 0$ M^{-1}). All divalent cations produce a larger effect on the zeta potential than predicted by this "screening" curve. In terms of the model they adsorb significantly to the PS molecules. For each of the divalent cations illustrated in Fig. 1, the experimentally observed zeta potentials may be adequately described by the simple Stern equation, Eqs. 3, 4, and 10, if we assume that the divalent cation forms only 1:1 complexes with PS.[7] Although this does not rule out the possibility that some 2:1 complexes are formed between phosphtidylserine and divalent cations, it is difficult to think of phenomena other than the formation of 1:1 complexes that would cause the zeta potential of the PS vesicles to reverse sign and become positive: if divalent cations form only 2:1 complexes with PS, the curves in Fig. 1 would asymptote zero potential at high concentrations of divalent cations.

There is a simple relationship between the binding constant K_2 (Eq. 6) and

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[6] Note that in the absence of divalent cations the zeta potentials do not differ significantly from −62.5 mV, the value predicted from the Stern equation if the intrinsic association constant of sodium with phosphatidylserine, K_1, is 0.6 M^{-1} and the hydrodynamic plane of shear is 2 Å from the surface of the membrane (Eisenberg et al., 1979). Note also that the addition of EDTA to the solution (filled symbols in Fig. 1) produces no significant change in the zeta potentials, indicating that the solutions and the lipid contain a negligible level of multivalent cationic contaminants.

[7] With the divalent cation UO$_2$ there appears to be a break in the curve in the vicinity of [UO$_2$] = 5 10^{-4} M, the concentration at which the zeta potential is zero. For 5 10^{-5} < [UO$_2$] < 2 10^{-4} the data are consistent with $K_2 = 10^3$ M^{-1}, whereas for 10^{-3} < [UO$_2$] < 2 10^{-2} M the data are consistent with $K_2 = 3 \times 10^3$ M^{-1}.
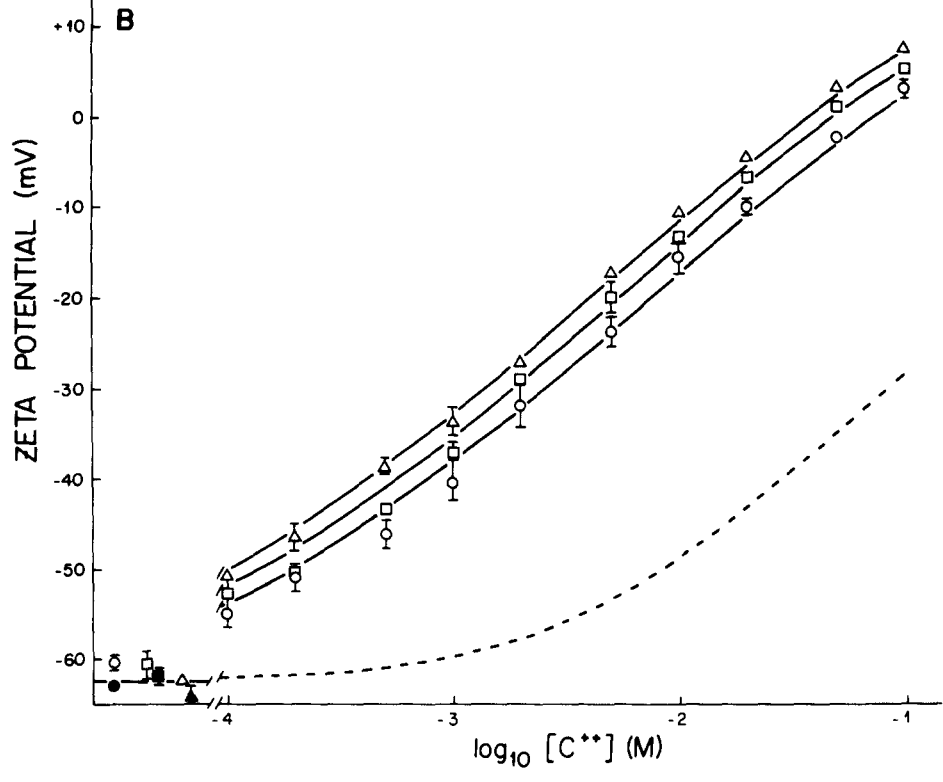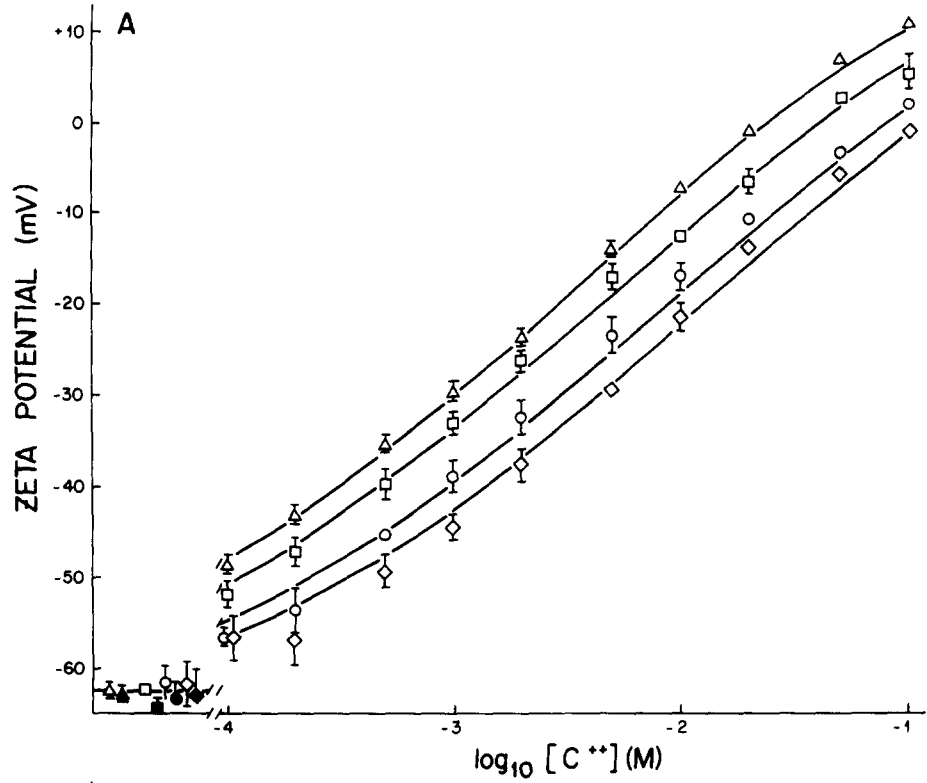
the bulk concentration of divalent cations at which the mobility reverses sign, C_{rev}^{++}. When the mobility is zero the zeta potential, ζ, surface potential, ψ_o, and charge density, σ, are also zero. If the membrane consists of a single species of negative lipid, Eqs. 10 and 4 predict that $K_2 = 1/C_{rev}^{++}$. For example, the zeta potential of a PS vesicle reverses sign in 0.08 M Ca (Fig. 1), which implies that the intrinsic association constant for the 1:1 complex is 12 M^{-1}. The use of this "reversal of charge" criterion to calculate the binding constant K_2 has several advantages: the calculation is independent of the number of monovalent cations adsorbed in electroneutral 1:1 complexes with PS, the number of divalent cations adsorbed in electroneutral 2:1 complexes with PS, the distance of the plane of shear from the membrane, the area of the lipid, any variation in the viscosity and dielectric constant of the aqueous phase adjacent to the membrane (Eq. 2), and most of the assumptions[8] inherent in the classic theory of the diffuse double layer, which are discussed elsewhere (Grahame, 1947; Verwey and Overbeek, 1948; Haydon, 1964; Mohilner, 1966; Barlow, 1970; Bockris and Reddy, 1973; Sparnaay, 1972; Aveyard and Haydon, 1973; McLaughlin, 1977).

On the other hand, the fit of the theory to the data obtained at other concentrations of divalent cations does depend on the values assumed for the monovalent cation binding constant ($K_1 = 0.6$ M^{-1}), the number of 2:1 complexes that PS forms with the divalent cation (none), and the distance of the plane of shear from the membrane (2 Å).

It clearly is desirable to make independent measurements of the number of adsorbed cations. We have done this with calcium. When the free concentration of calcium ($[Ca]^f$) in a solution of vesicles is low ($[Ca]^f < 50$ μM for [NaCl] = 0.1 M), it can be demonstrated, in terms of the Gouy-Chapman-Stern theory outlined above, that the excess number of divalent cations in the diffuse double layer per unit area of membrane, $C^{++} \int_0^\infty (\exp(-2F\psi(x)/RT) - 1)\, dx$, is negligible in relation to the number of calcium ions bound to a unit area of membrane, $K_2\{P^-\}C^{++} \exp(-2F\psi_o/RT)$ (e.g., Puskin, 1977; Nir et al., 1978). A knowledge of the total concentration of calcium in a solution of vesicles, $[Ca]^{tot}$, and a measurement of the free concentration of calcium with a calcium-sensitive electrode allows the bound concentration, $[Ca]^b$, to be calculated directly:

$$[Ca]^b = [Ca]^{tot} - [Ca]^f. \tag{11}$$

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[8] It is necessary to assume that discreteness of charge effects may be ignored (see footnote 2) and that the anion, chloride in our case, does not adsorb to the membrane.

FIGURE 1. The effect of divalent cations on the zeta potentials of multilamellar PS vesicles. The aqueous solutions contain 0.1 M NaCl buffered to pH 7.4 at 25°C with 0.001 M MOPS and either 0.0001 M EDTA (filled symbols) or the indicated concentration of divalent cation. The points indicate the average ($\pm$SD) of measurements on at least 20 vesicles in two separate experiments. The lines represent the least squares best fits of the Stern equation (Eqs. 3, 4, and 10), which were obtained with the following values of K_2, the intrinsic association constant of the divalent cation with PS (Eq. 6). (*A*) $\triangle$ Ni, 40 M^{-1}; $\square$ Mn, 25 M^{-1}; $\bigcirc$ Ca, 12 M^{-1}; $\lozenge$ Mg, 8 M^{-1}. (*B*) $\triangle$ Co, 28 M^{-1}; $\square$ Ba, 20 M^{-1}; $\bigcirc$ Sr, 14 M^{-1}.

The apparent association constant is defined as

$$K_A = \frac{\{CaPS\}}{[Ca]^f\{PS\}} = \frac{[Ca]^b}{[Ca]^f[PS]},$$ (12)

where the braces denote a surface concentration. [PS] is related to the total concentration of PS in the solution $[PS]^{tot}$, which is determined from phosphate analysis of the sonicated vesicle solution, by the expression

$$[PS] = \frac{\alpha[PS]^{tot}}{(1 + K_1 C^+ \exp[-F\psi_o/RT])} = 0.27\ [PS]^{tot}.$$ (13)

The factor α arises because only this fraction of the PS molecules is on the outer surfaces of the sonicated vesicles and, therefore, accessible to the calcium ions added to the solution. Our nuclear magnetic resonance (NMR) measurements indicate that $\alpha = \frac{2}{3}$. Similar values are observed for sonicated PC (Huang and Mason, 1978) and PG vesicles.[9] The denominator of Eq. 13 accounts for the number of PS molecules unavailable to bind Ca because of adsorbed sodium ions. The value of K_1 (Eq. 5) is 0.6 M^{-1}, and the value of ψ_o is -83 mV for PS in 0.1 M NaCl (Eisenberg et al., 1979). The value of the intrinsic association constant is calculated from K_A by means of Eq. 1. The values of K_2, as determined from the electrode measurements, were independent of calcium concentrations between 5 and 50 μM. The average value of K_2 was $11.4 \pm 2.7\ M^{-1}$ ($\pm$SD, $n = 23$). This number agrees within experimental error with the number calculated from the microelectrophoresis results presented in Fig. 1. Electrode measurements revealed that there is no significant variation in the ability of PS vesicles to bind Ca between pH 6.5 and 7.5, a result confirmed with electrophoresis measurements. Experiments with a pH electrode revealed that less than one proton is released from the PS membrane for every 100 calcium ions bound to the surface at pH 7.5, $T = 25°C$, a result that agrees qualitatively with the measurements of Puskin and Coene (1980). Our conclusion from the electrode and electrophoresis experiments is that the simple Stern equation can describe the essential features of the adsorption of the alkaline earth cations to bilayer membranes formed from bovine brain PS.

We also made NMR measurements of the binding of cobalt to PS membranes. The number of phosphate groups involved in inner-sphere complexes with cobalt can be determined from the line width of the ^{31}P NMR signal obtained from sonicated PS vesicles. Under conditions where most of the added cobalt is bound to the PS membranes, only about one-tenth of the bound cobalt ions are involved in inner-sphere complexes with the phosphate group. ^{13}C NMR experiments with PS vesicles demonstrate that a larger fraction of the cobalt ions form inner-sphere complexes with the carboxyl group. The remainder, up to one-half the bound cobalt ions, presumably form outer-sphere complexes with PS.

[9] McLaughlin, A., and S. McLaughlin. Unpublished observations.

Measurements with a calcium-sensitive electrode reveal that there is little temperature dependence of the binding of calcium to sonicated PS vesicles. Zeta potential measurements can also be used to provide qualitative information about the temperature dependence of the binding of a charged ligand to a lipid vesicle. Fig. 2 illustrates that the zeta potentials of PS vesicles formed in 0.1 M NaCl are essentially independent of temperature in the presence of either 0.5 or 5 mM calcium. The largest dependence of zeta potential on temperature was observed in the absence of calcium; in this case, the magnitude of the zeta potential decreased 10 mV, from about −60 to −50 mV, as the temperature was lowered from 25° to 5°C (Fig. 2). The cause of this
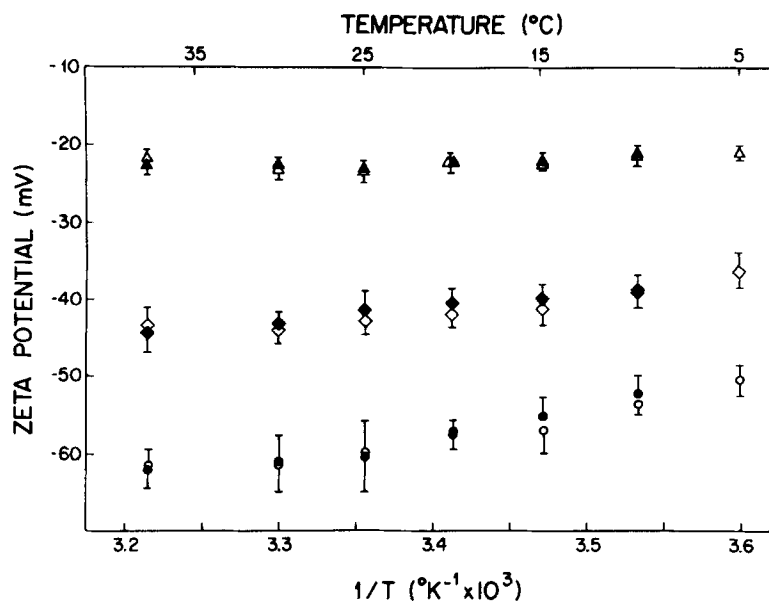

FIGURE 2.   The effect of temperature on the zeta potential of multilamellar PS vesicles. The PS vesicles were formed in 0.1 M NaCl buffered to pH 7.5 with 0.001 M MOPS, calcium was added to a level of 0 (circles), 0.5 (diamonds), or 5 mM (triangles), measurements were made at 38°C (open symbols), and the temperature was lowered to 30°C, then in 5° steps to 5°C, and, finally (closed symbols), raised again in steps to 38°C.

change is not known, but a similar decrease of ~10 mV in the magnitude of the zeta potential of dipalmitoylphosphatidylserine (MacDonald et al., 1976), dimyristoylphosphatidylserine,[9] and dimyristoylphosphatidylgylcerol[9] vesicles occurs when the temperature is lowered below T_c, the transition temperature. The transition from a "liquid crystalline" to a "gel" phase occurs in the vicinity of 10°C for vesicles formed from brain PS in the absence of calcium; the transition temperature increases in the presence of calcium (Jacobson and Papahadjopoulos, 1975). Thus, Fig. 2 illustrates both that the temperature dependence of the binding of Ca to PS is small and that the binding of calcium to PS does not increase significantly when the temperature is lowered

below T_c. This is also true for vesicles formed from dimyristoylphosphatidyl-serine, as revealed by both electrode and electrophoresis measurements.[9] At 25°C, where dimyristoylphosphatidylserine exists in a gel state, the intrinsic association constants for Ca and Mg as determined from charge reversal measurements are 17 and 11 M^{-1}, respectively.[9] These values are only 40% higher than the binding constants of Ca and Mg with brain PS (Fig. 1), which exists in the liquid crystalline state at 25°C.

PC and PE Membranes

Many biological membranes contain 10–20% PS and a higher concentration of the zwitterionic lipids phosphatidylcholine (PC) and phosphatidylethanol-amine (PE). Fig. 3 illustrates the effects of calcium and magnesium on the zeta potentials of vesicles formed with egg PC (open symbols) and bacterial PE (filled symbols). The results obtained with PC are similar to those reported previously (McLaughlin et al., 1978).[10] The data for Ca and Mg can be well described by the Stern equation, with intrinsic association constants, K_3, of ~3 and 2 M^{-1} for Ca and Mg, respectively. The value for the association constant of Ca that we deduced from zeta potential measurements (Fig. 3) agrees with the value Grasdalen et al. (1977) deduced from NMR measurements (2.2 M^{-1}). The results we obtained with vesicles formed from PE are similar to those obtained with vesicles formed from PC.

PC:PS Membranes

We now question whether the adsorption of Ca to a membrane composed of a mixture of the negative lipid PS and a zwitterionic lipid can be predicted from the ability of Ca to adsorb to the individual lipid components. Fig. 4 illustrates the effect of Ca on the zeta potential of vesicles formed from mixtures of PS and PC. The data can be adequately described by the Stern equation with the same values of the parameters that were used to describe the adsorption of Ca to PS and to PC (K_1 = 0.6, K_2 = 12, K_3 = 3 M^{-1}). A reasonable fit to the zeta potential data obtained with Mg and PC:PS vesicles was also observed (data not shown) with the same binding constants that were obtained from a study of the ability of Mg to adsorb to PS (Fig. 1) and PC (Fig. 3) vesicles.

The Stern equation describes the data obtained both when PC:PS vesicles are formed in decimolar solutions of sodium (Fig. 4), which does adsorb significantly to PS, and when PC:PS vesicles are formed in cesium (Fig. 5), which does not adsorb strongly to PS (Eisenberg et al., 1979). The fit is not perfect but it is adequate.

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[10] The measurements were repeated because the degree of calcium binding to PC depends on the area the lipid occupies in the bilayer (Lau et al, 1979; Lis et al., 1979). Egg PC is not well defined with respect to the composition of the fatty acid chains, which determine the area, and we wanted to measure the binding constant of calcium to egg PC on the same sample of lipid used for experiments with mixtures of PS. The values of the binding constants calculated here are slightly higher than those previously reported because, in the fit of the Stern equation to the data in Fig. 3, we assume that the plane of shear is 2 Å from the surface of the membrane (Eisenberg, et al., 1979), whereas the previous data were interpreted by assuming that the binding plane and the plane of shear were coincidental.

PE:PS Membranes

There is reasonably strong evidence that PS is located mainly in conjunction with PE in the inner monolayer of erythrocyte membranes (e.g., Rothman and Lenard, 1977; Op den Kamp, 1979). Although there is some controversy about the location of PE and PS in retinal rod outer segment disk membranes (Drenthe et al., 1980), evidence from chemical labeling experiments suggests
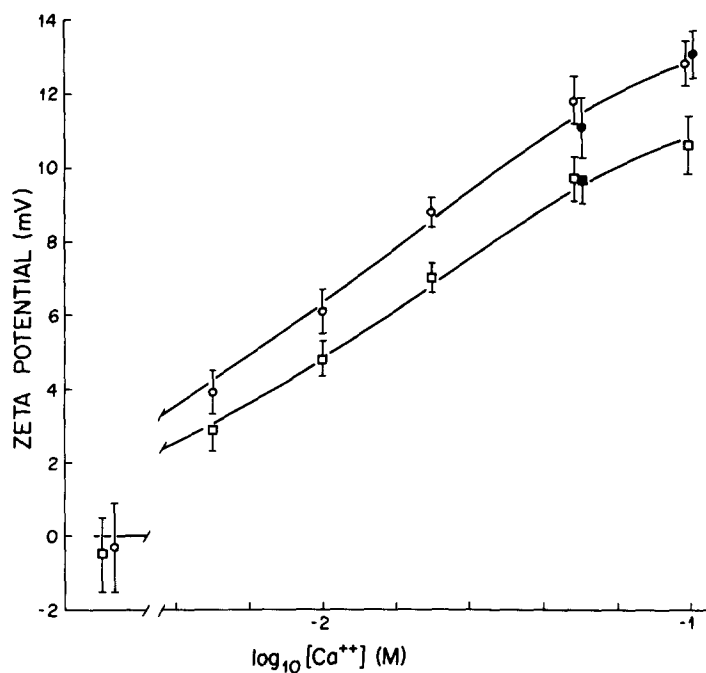

FIGURE 3.   The effects of calcium (open circles) and magnesium (open squares) on the zeta potentials of vesicles formed from egg phosphatidylcholine (PC). The aqueous solutions contain 0.1 M NaCl, 0.001 M MOPS, pH 7.5, $T = 25°C$. The vertical lines through the points indicate the SD of measurements made on 30 vesicles in three separate experiments. The curves are the prediction of the Stern equation, drawn to give a least squares best fit to the value of K_3, the 1:1 association constant of the divalent cation with PC: $K_3 = 2.7 \ M^{-1}$ for Ca, 1.8 M^{-1} for Mg. The effects of calcium (filled circles) and magnesium (filled square) on the zeta potentials of bacterial phophatidylethanolamine vesicles are similar to their effects on PC vesicles.

that these two lipids are preferentially located on the outer or cytoplasmic monolayer of the disks (Raubach et al., 1974; Smith et al., 1977; Crain et al., 1978; Dratz et al., 1979). The adsorption of Ca to PE:PS membranes is, therefore, of some biological interest. Although Ca binds equally well to vesicles formed from either PE or PC (Fig. 3), it cannot be assumed that these two lipids will behave in an identical manner when mixed with PS. Recent experiments have demonstrated that calcium can induce the formation of

nonbilayer (hexagonal H_{II}) regions in 4:1 PE:PS unsonicated vesicles (Cullis and Verkleij, 1979). In spite of the ability of PE to exist in nonbilayer configurations (Cullis and de Kruijff, 1978 and 1979), Ca adsorbs to multilamellar PE:PS vesicles exactly as predicted by the simple Gouy-Chapman-Stern model. Fig. 6 illustrates that the zeta potential data obtained with PE:PS vesicles do not differ significantly from those obtained with PC:PS vesicles (Fig. 4). Both sets of data can be described by the Stern equation with K_1 =
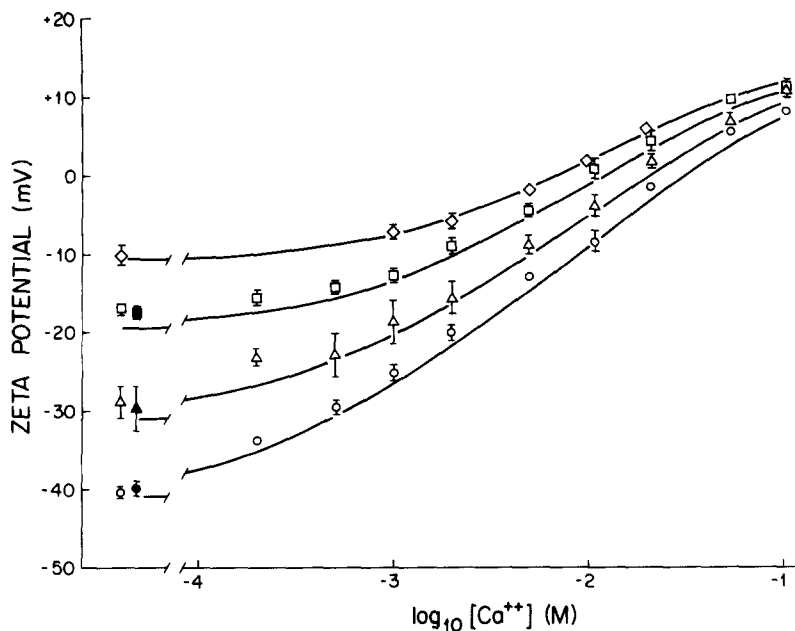
FIGURE 4.   The effect of calcium on the zeta potentials of vesicles formed from mixtures of egg PC and brain PS for PC:PS ratios of 20:1, ◇; 10:1, □; 5:1, △; 2.5:1, ○. The aqueous solutions contained 0.1 M NaCl, 0.001 M MOPS buffered to pH 7.4 at 25°C and either 0.0001 M EDTA (filled symbols) or the indicated concentration of calcium. The curves are the predictions of the Stern equation with K_1 = 0.6 M^{-1}, K_2 = 12 M^{-1}, and K_3 = 3 M^{-1}. The plane of shear is assumed to be 2 Å from the surface of the membrane and each lipid is assumed to occupy an area of 70 Å^2. The vertical bars through the experimental points indicate the standard deviations of the 20 measurements.

0.6, K_2 = 12, and K_3 = 3 M^{-1} and are, therefore, consistent with our assumption that calcium forms mainly 1:1 complexes with PS. The fit of the theoretical curves to the data was equally good when the PE:PS vesicles were formed from egg rather than bacterial phosphatidylethanolamine (data not shown).

We also studied the temperature dependence of the binding of calcium to 5:1 PE:PS vesicles formed in either decimolar NaCl or KCl. The zeta potential vs. [Ca] curves obtained at 15° and 35°C are identical, within experimental

error, to those obtained at 25°C (Figs. 6 and 7). These results (data not shown) are consistent with the observation that there is little temperature dependence of the binding of calcium to PS (Fig. 2). We examined the adsorption of calcium to PE:PS vesicles in the presence of 0.15 M KCl because potassium is a major intracellular cation. The fit of the Stern equation to the data
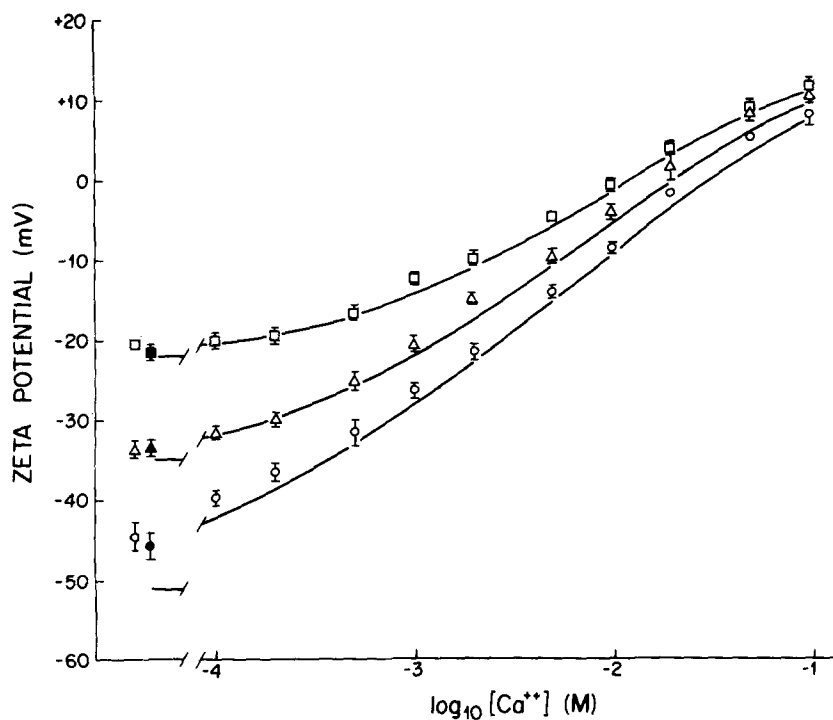

FIGURE 5.   The effect of calcium on the zeta potential of PC:PS vesicles formed in CsCl. The vesicles were formed from mixtures of egg PC and brain PS in the ratios 10:1, □; 5:1, △; 2.5:1, ○. The aqueous solutions contained 0.1 M CsCl, 0.001 M MOPS buffered to pH 7.4 at 25°C and either 0.0001 M EDTA (filled symbols) or the indicated concentrations of calcium. The curves are the predictions of the Stern equation with $K_1 = 0.05$ M^{-1} (Eisenberg et al., 1979), $K_2 = 12$ M^{-1}, and $K_3 = 3$ M^{-1}. The plane of shear is assumed to be 2 Å from the surface of the membrane, and each lipid is assumed to occupy an area of 70 Å^2. The vertical bars through the points indicate the standard deviations of 20 measurements.

presented in Fig. 7 is acceptable. The values of the parameters used in the Stern equation were $K_1 = 0.15$ M^{-1} (Eisenberg et al., 1979), $K_2 = 12$ M^{-1} (Fig. 1), and $K_3 = 3$ M^{-1} (Fig. 3).

GMO:PS Membranes

The data obtained with single multilamellar vesicles formed from mixtures of phospholipids can all be understood in terms of the abilities of the individual

phospholipids to adsorb ions. This is not true when vesicles are formed from mixtures of PS and the neutral lipid glycerolmonooleate (GMO). It is apparent from the lower curve in Fig. 7 that calcium does not adsorb as strongly to vesicles formed from 30% (by weight) PS in GMO as it does to vesicles formed from 30% (by weight) PS in PE. The greater effect of calcium on vesicles formed from PE:PS mixtures can be partly, but not completely, explained by the ability of calcium to adsorb to PE (Fig. 3) but not to GMO. The zeta potential of GMO vesicles is zero, independent of the concentration of calcium
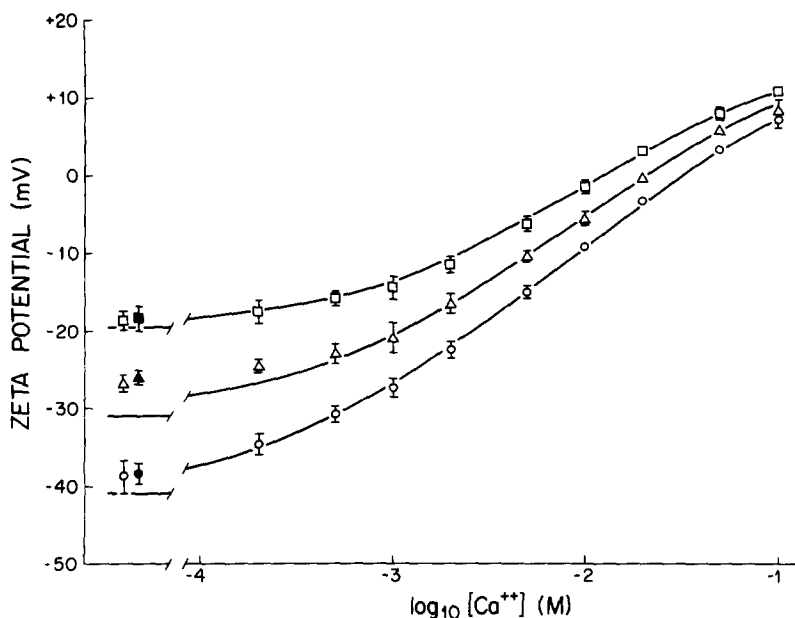

FIGURE 6.   The effect of calcium on the zeta potentials of vesicles fromed from mixtures of bacterial PE and brain PS for PE:PS ratios of: 10:1, □; 5:1, △; 2.5:1, ○. The aqueous solutions contained 0.1 M NaCl, 0.001 M MOPS buffered to pH 7.5 at 25°C and either 0.0001 M EDTA (filled symbols) or the indicated concentration of calcium. The curves are the predictions of the Stern equation with $K_1 = 0.6$ M^{-1}, $K_2 = 12$ M^{-1}, and $K_3 = 2$ M^{-1}. The plane of shear is assumed to be 2 Å from the surface of the membrane and each lipid is assumed to occupy an area of 70 Å^2.

(data not shown). In terms of our model, the Stern equation with $K_1 = 0.15$, $K_2 = 12$, and $K_3 = 0$ M^{-1} should fit the GMO:PS data. In fact, the data are described by the Stern equation only if a weaker association of Ca with PS is assumed, $K_2 = 5$ M^{-1} (Fig. 7).

The experiments illustrated in Fig. 8 were designed to examine in more detail the adsorption of calcium to membranes formed from mixtures of GMO and PS. The association constant of the Ca-PS complex must be assumed to decrease as the surface concentration of PS decreases upon dilution with

GMO if the data are to be described by the Stern equation. The reason for this decrease, if real, is not understood.

Effect of Varying [NaCl]

The Gouy-Chapman-Stern theory presented above predicts that a 10-fold decrease in the concentration of monovalent ions will produce a change of about −60 mV in the surface potential, ψ_0, of a PS membrane. This prediction is independent of the degree of binding of the monovalent cation to the membrane, at least in the range encountered with the alkali metal cations (0 < K_1 < 1 M^{-1}; Eisenberg et al., 1979). If a divalent cation is present in the

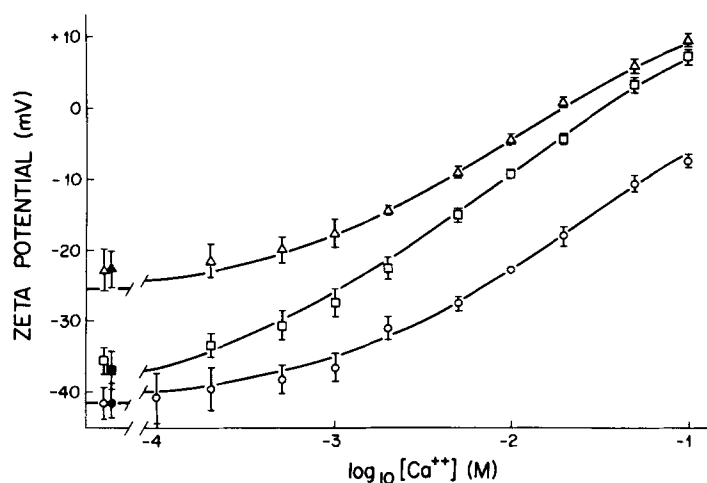Figure 7. The effect of calcium on the zeta potentials of vesicles formed from 5.6:1, △, and 2.3:1, □, PE:PS mixtures and a 2.3:1 GMO:PS, ○, mixture. The aqueous solutions contained 0.15 M KCl buffered to pH 7.5 with 0.001 M MOPS at 25°C and either 0.0001 M EDTA (filled symbols) or the indicated concentration of calcium ions. The curves are the predictions of the Stern equation for K_1 = 0.15 M^{-1}, K_2 = 12 M^{-1}, and K_3 = 3 M^{-1} (upper two curves, PE:PS mixtures) and K_1 = 0.15 M^{-1}, K_2 = 5 M^{-1}, and K_3 = 0 M^{-1} (lower curve, GMO:PS mixture).

aqueous phase at a concentration low enough that it does not affect ψ_0, the Boltzmann equation predicts that the surface concentration of the divalent cation will be increased by about two orders of magnitude when [NaCl] is decreased from 0.1 to 0.01 M. Our experiments with calcium electrodes (data not shown) and the dialysis experiments of Nir et al. (1978) demonstrate that the apparent association constant of calcium with PS indeed increases about two orders of magnitude when [NaCl] decreases from 0.1 to 0.01 M. Another intuitively apparent prediction relates to the concentration of calcium that produces a measurable effect on the surface or zeta potential. This concentration should decrease by about two orders of magnitude when [NaCl] is lowered

from 0.1 to 0.01 M. Many experiments have demonstrated that this prediction is qualitatively true (e.g., McLaughlin, 1977; Ohki and Sauve, 1978). The results presented in Fig. 9 also agree with this prediction. A concentration of 10^{-3} M Ca produces a change of ~25 mV in the zeta potential in 0.1 M NaCl (Fig. 1), whereas a concentration of 10^{-5} M Ca produces a similar change in
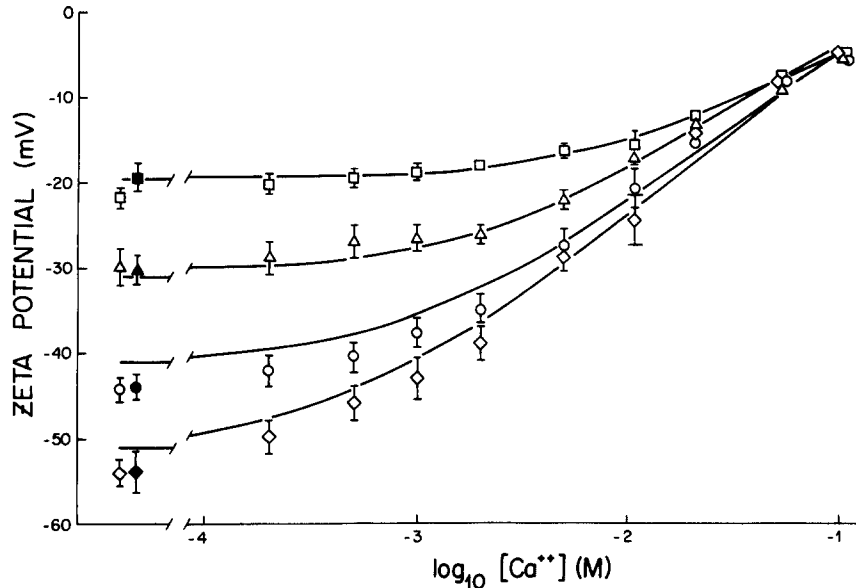

FIGURE 8.   The effect of calcium on the zeta potentials of vesicles formed from mixtures of GMO and brain PS. The GMO:PS ratios were, by weight; 10:1, □; 5:1, △; 2.5:1, ○; 1:1, ◇. The aqueous solutions contained 0.1 M NaCl buffered to pH 7.4 at 25°C with 0.001 M MOPS and either 0.0001 EDTA (filled symbols) or the indicated concentrations of calcium, which was added as the chloride salt to the solution containing the vesicles. The vertical lines through the experimental points are the standard deviations of the 20 experimental measurements. The data cannot be adequately described by the Stern equation with $K_1 = 0.6$, $K_2 = 12$, $K_3 = 0$ M^{-1}. A better fit is obtained, as indicated by the curves, if one assumes that the association constant of the Ca-PS complex decreases as the surface concentration of PS decreases. The curves were drawn according to the Stern equation with $K_1 = 0.6$ M^{-1}, $K_3 = 0$ M^{-1}, and $K_2 = 5$, ◇; 4, ○; 4, △; and 1.5, □ M^{-1}. It is assumed that PS and GMO occupy areas of 70 and 35 Å^2, respectively.

the zeta potential in 0.01 M NaCl (Fig. 9). A more detailed analysis of our data, however, indicates that the intrinsic binding constant of the Ca-PS complex appears to have increased by a factor of 3. Specifically, the best fit is obtained with $K_2 = 36$ M^{-1} in 0.01 M NaCl (Fig. 9), whereas the best fit in 0.1 M NaCl was obtained with $K_2 = 12$ M^{-1} (Fig. 1). The binding constant
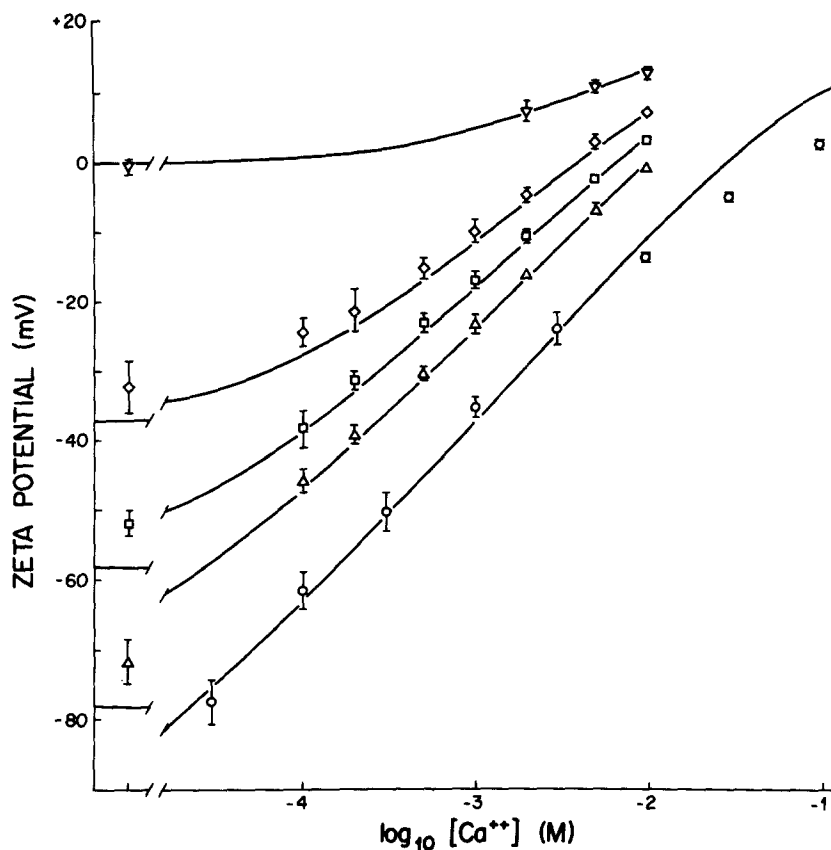
FIGURE 9.   The effect of calcium on the zeta potential of vesicles formed from egg PC, brain PS, and PC:PS mixtures in 0.01 M NaCl. The PC:PS ratios were, by weight: 1:0, ∇; 20:1, $\Diamond$; 10:1, $\square$; 5:1, $\triangle$; 0:1, $\bigcirc$. The aqueous solutions contained 0.01 M NaCl buffered to pH 7.5 with 0.001 M MOPS at 25°C. The vertical lines through the experimental points indicate the standard deviations of the 20 experimental measurements. A least squares best fit of the Stern equation was obtained for the PC vesicles with $K_3 = 5$ M^{-1} and for the PS vesicles for $K_2 = 36$ M^{-1}, assuming $K_1 = 0.6$ M^{-1}. The curves describing the data obtained with PC:PS mixtures are drawn for $K_1 = 0.6$, $K_2 = 36$, $K_3 = 5$ M^{-1}. The zeta potential of PS vesicles in the absence of calcium was -110 ± 9 mV, which is close to the theoretical value of -118 mV predicted from the Stern equation with $K_1 = 0.6$ M^{-1}.

of Ca with PC also increases somewhat: from 3 M^{-1} in 0.1 M NaCl (Fig. 3) to 5 M^{-1} in 0.01 M NaCl (Fig. 9). Fig. 9 illustrates that the effects of calcium on the zeta potentials of vesicles formed from mixtures of PC and PS in 0.01 NaCl can all be adequately described by the Stern equation with these values of the calcium binding constants. The apparent increase in the binding constant of the divalent cation for PC and PS observed with a decrease in the [NaCl] is not restricted to Ca; a similar change was noted for Mg (data not

shown). This phenomenon is also observed when the vesicles are formed from egg phosphatidylglycerol (data not shown). The cause of this relatively minor deviation from the model presented above is not well understood at the present time.

Aggregation of Vesicles and Limitations on the Use of the Stern Equation

Although no theory is available at the present time to describe the adsorption of divalent cations to phospholipid vesicles when aggregation occurs, calcium electrodes can be used to document some intriguing phenomena. When the free Ca concentration ($[Ca]^f$) in a solution of sonicated PS vesicles in low ($[Ca]^f < 0.2$ mM), the degree to which Ca adsorbs to PS can be well described by the Stern equation and is independent of time, at least for $1 < t < 30$ min (squares and triangles in Fig. 10). The Stern equation predicts that less Ca should be bound in the presence of Mg, because Mg will both lower the magnitude of the surface potential and occupy binding sites. However, when 5 mM magnesium is added to the solution of PS vesicles before the addition of Ca, an interesting effect occurs that has been described in some detail by Papahadjopoulos and his co-workers (Portis et al., 1979). In the presence of Mg, $[Ca]^f$ actually decreases to a value significantly lower than that observed without Mg (circles in Fig. 10). Aggregation of the vesicles occurs concomitantly with the decrease in $[Ca]^f$; the turbidity of the solution increases markedly. The decrease in $[Ca]^f$ is critically dependent on the Mg concentration. It is not observed, at least over a 30-min time-course, in the presence of 1 instead of 5 mM Mg (data not shown). Under identical conditions, the decrease in $[Ca]^f$ is not observed with PG vesicles (data not shown). Finally, it is not observed with 2:1 PC:PS sonicated vesicles, even if the concentration of vesicles is increased 10-fold, and the Ca and Mg concentrations are increased four-fold (data not shown).

DISCUSSION

Our major conclusion is that the adsorption of divalent cations to phospholipid bilayer membranes containing brain phosphatidylserine can be described surprisingly well by the simplest form of the Gouy-Chapman-Stern theory. To test the Stern equation one must measure both the electrostatic potential and the number of absorbed divalent cations. The zeta potential results with PS vesicles (Fig. 1) suggest that $K_2 = 12$ M^{-1} for Ca; the electrode measurements suggest that $K_2 = 11 \pm 3$ M^{-1}. The use of a calcium-sensitive electrode is similar in principle to the use of electron spin resonance to measure the free concentration of Mn (Puskin, 1977; Puskin and Martin, 1979; Puskin and Coene, 1980), and the use of equilibrium dialysis to determine the free concentration of Ca and Mg (Nir et al., 1978; Newton et al., 1978; Portis et al., 1979) in a solution of PS vesicles. Puskin (1977) measured an apparent association constant of $1.4 \ 10^4$ M^{-1} for Mn to bovine PS in 0.1 M NaCl. If we correct for the sites unavailable because of sodium binding by taking $K_1 = 0.6$ M^{-1} (Eisenberg et al., 1979) the intrinsic association constant (Eqs. 1, 12, and 13 with $\psi_o = -83$ mV) becomes $K_1 \cong 50$ M^{-1}. This number agrees within a factor of 2 with the value of 25 M^{-1} deduced from our zeta potential

measurements (Fig. 1). The most accurate equilibrium dialysis results available (Portis et al., 1979) indicate that the intrinsic 1:1 association constants of Ca and Mg with PS, as calculated from the Stern equation, are 17 and 10 M^{-1}, respectively.[11] These numbers agree quite well with the values of 12 and 8 M^{-1} calculated from the zeta potential measurements (Fig. 1).
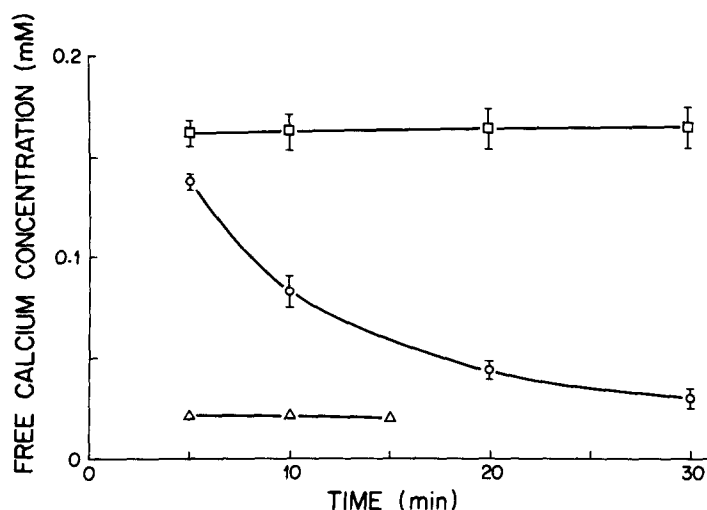

Figure 10. The "synergistic" effect of Mg on the binding of Ca to brain PS, which was present initially in the form of sonicated vesicles. The ordinate indicates the free concentration of Ca in the solution, $[Ca]^f$, as measured directly with a Ca-sensitive electrode. The abscissa indicates the time after the addition of $CaCl_2$ to the solution. All solutions contain 0.1 M NaCl buffered to pH 7.5 with either 0.01 or 0.001 M MOPS AT 22°C. The triangles illustrate the results obtained when 0.07 mM $CaCl_2$ is added to a solution containing 1.6 mM PS. The value of $[Ca]^f$ does not change with time: $[Ca]^f = 0.02$ mM, as expected from the Stern equation, assuming $K_1 = 0.6$, and $K_2 = 12$ M^{-1}. The squares indicate that the binding of Ca to PS also does not depend on time when 0.4 mM $CaCl_2$ is added to a solution containing 2.1 mM PS. $[Ca]^f$ remained constant at 0.16 mM for 30 min. The circles illustrate the results obtained when 5.1 mM $MgCl_2$ was added to a 2.1 mM solution before the addition of 0.4 mM $CaCl_2$. The Stern equation predicts that the apparent binding constant of Ca should decrease in the presence of Mg. In other words, $[Ca]^f$ should have been greater in the presence of Mg. In fact, when 5 mM Mg is present, $[Ca]^f$ falls to a much lower value than is predicted from the Stern equation. An increase in the turbidity of the solution is apparent to the eye as $[Ca]^f$ decreases. Other aspects of this phenomenon are discussed by Portis et al. (1979). The line connecting the circles has no theoretical significance.

[11] These numbers differ by a factor of two from the intrinsic association constants calculated from the same data using the Stern equation analysis of Nir et al. (1978), because it was assumed that a divalent cation combines with two rather than one PS and that the number of binding sites is half the number of free PS molecules. The values of $K_1 = 35$ and 20 M^{-1} calculated for Ca and Mg in this way (Portis et al., 1979) should thus be divided by two if the stoichimetry is actually 1:1, as we argue in this report.

The zeta potential results illustrated in Fig. 1 are also consistent with previous measurement of the calcium-induced change in electrostatic potential above a PS monolayer (Ohki and Sauve, 1978) or within a PS bilayer (McLaughlin et al., 1971). Both sets of data were originally described with the Stern equation by assuming (a) that the dipole potential did not change on addition of calcium, which allowed one to equate the measured change in potential with $\Delta\psi_o$, the change in potential in the aqueous phase at the interface, (b) that sodium did not adsorb to PS but exerted only a screening effect, and (c) that one calcium was bound to two PS molecules with the number of binding sites equal to one-half the number of free PS molecules and the intrinsic association constant $= 0.1$ M^{-1}. The monolayer data, which constitute the most direct measure of change in electrostatic potential, and the theoretical fit are illustrated in Fig. 11 (solid curve). The available evidence now suggests that sodium does absorb to PS, with an association constant of $K_1 = 0.6$ M^{-1} (Eisenberg et al., 1979), and that calcium forms 1:1 complexes with PS. Fig. 11 illustrates (dashed line) that the surface potential data can also be described by the Stern equation using these assumptions. A good fit was obtained with $K_2 = 6$ M^{-1}. This value of K_2 agrees, within a factor of 2, with the value obtained from the charge reversal and the electrode measurements. The association constants calculated for Mn and Mg from the data obtained by Ohki and Sauve (1978) also agree, within a factor of 2 and 3, respectively, with values calculated from charge reversal measurements (Fig. 1).

Cohen and Moronne[3] have recently made an extensive investigation of the ability of calcium and magnesium to affect the surface potential of planar black lipid membranes containing brain PS, utilizing the conductance probes monazomycin and valinomycin. They were also able to describe the data they obtained with the Stern equation, using association constants similar to those reported here.

The only results not agreeing with our own appear to be those of Barton (1968), who also made "charge reversal" microelectrophoresis measurements. He reported that the logarithm of the intrinsic association constant of calcium with PS is 4.07, a number that disagrees by three orders of magnitude with the number reported here. Inasmuch as no actual mobility or zeta potential data obtained in the presence of Ca are presented in his paper, a direct comparison of our data with his is not possible. The zeta potential data that we obtained with Ca and PS agree qualitatively with the data of Papahad-jopoulos (1968).

Although most features of the data that we and others have obtained with respect to the adsorption of the alkaline earth cations to single multilamellar vesicles, sonicated vesicles, planar black lipid membranes, and monolayers containing PS are remarkably consistent with the simple form of the Stern equation discussed above, we do not wish to imply that all alternative interpretations of the data can be ruled out. For example, our results demonstrate that some 1:1 complexes of bovine brain PS and Ca must be formed (Figs. 1 and 4–7) but do not rule out the possibility that some 2:1 complexes

are formed. They also do not rule out the possibility that anions might adsorb to lipids or to multivalent cations that are themselves adsorbed to phospholipids (Grasdalen et al., 1977; Westman and Eriksson, 1979). In spite of these and other reservations, we note that the Stern equation is the simplest
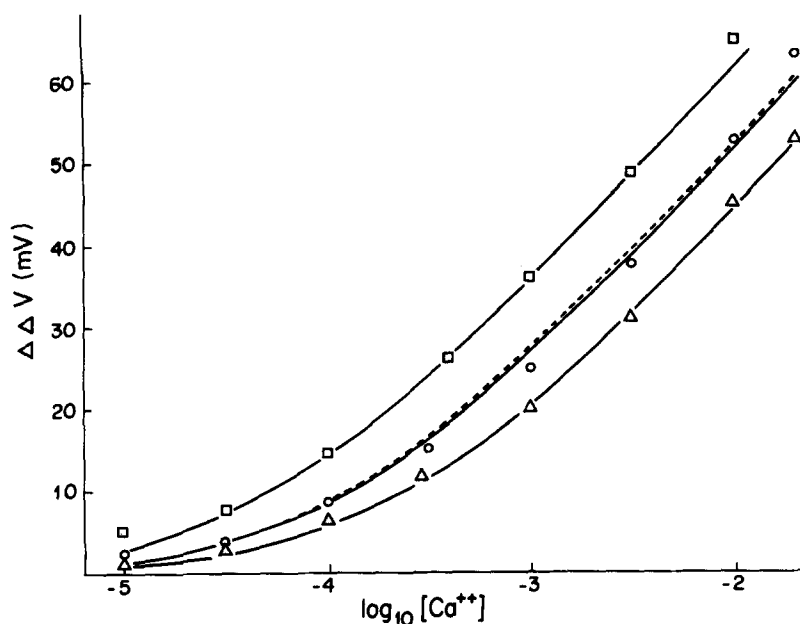

Figure 11. The changes in the surface potential above a PS monolayer (65 Å^2/molecule) measured directly by Ohki and Sauve (1978). The aqueous solutions contained the indicated concentrations of Mn (squares), Ca (circles), or Mg (triangles), as well as 0.09 M NaCl and 0.01 M tris (hydroxymethyl)aminomethane (Tris) at pH 7.4, $T = 21°C$. The data were originally described with the Stern equation by assuming that the monovalent cations do not absorb to PS and that Ca adsorbs to two PS molecules with an intrinsic binding constant of 0.1 M^{-1} (dashed line). Similar data obtained by McLaughlin et al. (1971) on PS bilayers using conductance probes were originally interpreted in an identical manner. The data can also be described by the Stern equation (Eqs. 3, 4, and 10) if it is assumed that sodium adsorbs to PS with an association constant $K_1 = 0.6$ M^{-1} and that Ca binds to one PS molecule with an intrinsic binding constant of $K_2 = 6$ M^{-1} (middle curve). The curves through the Mn and Mg points were drawn according to the Stern equation with $K_1 = 0.6$ M^{-1} and $K_2 = 15$ and 3 M^{-1}, respectively. These numbers agree, within a factor of 3, with the association constants deduced from charge reversal measurements on multilamellar vesicles (Fig. 1).

theoretical formulation capable of providing a good description of the existing experimental results. The biologically significant conclusion to be drawn from this study is that the Stern equation can be used with some confidence to describe the adsorption of the alkaline earth cations to either artificial phos-

pholipid bilayer membranes or the bilayer component of biological membranes that contain phosphatidylserine as their major negatively charged lipid. We stress, however, that it would not be appropriate for us to attempt to use the Stern equation to describe the adsorption of divalent cations to aggregates of vesicles: the aqueous diffuse double layers of the bilayers in these aggregates will overlap and interact with one another. This consideration is particularly important in the case of calcium, which causes PS vesicles to fuse and form structures that have been termed "cochleate" cylinders (Papahadjopoulos et al., 1975). Water may be essentially excluded from between the bilayers in these cylinders: when there is no aqueous phase there clearly cannot be an aqueous diffuse double layer. Furthermore, if the addition of calcium "results in a close apposition of the bilayers, essentially free of interlamellar water" (Portis et al., 1979) and if calcium is essentially the only ion that binds to PS under these conditions, the principle of electroneutrality requires that the effective stoichiometry be one calcium ion for every two negatively charged phospholipid molecules. Thus, there is not necessarily any contradiction between our results (Fig. 1), which suggest a 1:1 stoichiometry for the adsorption of Ca to PS in unaggregated vesicles, and the equilibrium dialysis results of Newton et al. (1978) and Portis et al. (1979), which suggest that when the Ca concentration is greater than $\sim$ 1 mM, the stoichiometry in the cochleate cylinders is one calcium for every two PS molecules. There also is not necessarily any contradiction between our observation that calcium adsorbs equally well to unaggregated multilamellar vesicles formed from mixtures of either PE or PC and PS (Figs. 4 and 6), our implicit assumption being that the lipids in these vesicles are in a bilayer configuration, and the ^{31}P NMR observations of Cullis and Verkleij (1979). They demonstrated that 15 mM calcium produces nonbilayer (hexagonal H_{II}) structures in membranes composed of 4:1 PE:PS mixtures. Concentrations of calcium >1 mM, however, cause gross aggregation of 4:1 PE:PS vesicles.[12] The possibility that Ca can induce nonbilayer structures in PE:PS vesicles only when the vesicles aggregate is certainly speculative and requires further experimental support, but could reconcile the two sets of experimental results.

The need for new theoretical formulations to explain the adsorption of divalent cations to membranes that are closely apposed is illustrated by the ability of Mg to exert a "synergistic" effect on the adsorption of Ca when aggregation of the PS vesicles occurs (Portis et al., 1979; Fig. 10), a phenomenon that is not predicted by the Stern equation.

Another unexplained phenomenon manifests itself when PS is mixed with zwitterionic phospholipids. Vesicles formed from a given ratio of either PC or PE and PS adsorb Ca equally well (Figs. 4 and 6) but have dramatically different aggregation properties. Sonicated PS vesicles formed in 0.1 M NaCl aggregated at calcium concentrations >1 mM (e.g., Sun et al., 1978), vesicles formed from a 1:1 PC:PS mixture require $\sim$10 mM Ca for significant aggregation (e.g., Ohki and Duzgunes, 1979), and vesicles formed from a 4:1 PC:PS mixture do not aggregate at Ca concentrations < 100 mM.[12] On the

[12] Unpublished observation.

other hand, 1 mM Ca (or 2mM Mg) is quite sufficient to produce a marked aggregation of both 2.5:1 and 4:1 sonicated PE:PS vesicles.[12] Kolber and Haynes (1979) also stressed the role that PE plays in membrane aggregation and suggested that "aggregation occurs due to intervesicular head-to-tail binding of PE molecules." In our opinion, the detailed molecular mechanisms responsible for vesicle aggregation are not well understood.

Finally, we are interested in the relative ability of divalent cations to bind to artificial bilayer membranes and to shift the conductance-voltage curves of ionic channels in biological membranes. The selectivity sequence for PC is Mn > Ca ≈ Co ≈ Mg ≈ Ni > Sr > Ba (McLaughlin et al., 1978). Under identical conditions the sequence for PS is Ni > Co > Mn ≈ Ba ≈ Sr ≈ Ca > Mg (Fig. 1). As expected, the sequence for a membrane formed from a 2.5: 1 mixture of PC and PS in 0.1 M NaCl, 0.001 M 3-(N-morpholino)-propane sulfonic acid (MOPS), pH 7.5, $T = 25°C$, lies between these two sequences. Specifically, we found that Mn ≈ Ni > Co > Ca > Mg ≈ Ba ≈ Sr (data not shown). This is close to the sequence in which these cations shift the conductance-voltage curves of the sodium channel in myelinated nerve: Ni > Co ≈ Mn > Ca > Mg > Ba ≈ Sr (Hille et al., 1975). This result provides no real evidence that PS is responsible for the charges adjacent to the sodium channel, but a negative result would have argued against the possibility.

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