J. Membrane Biol. 76, 183–193 (1983)

The Journal of
Membrane Biology

Dimethonium, A Divalent Cation that Exerts Only a Screening Effect on the Electrostatic Potential Adjacent to Negatively Charged Phospholipid Bilayer Membranes

Alan McLaughlin*, Wai-Kwong Eng, Gerard Vaio, Tazewell Wilson, and Stuart McLaughlin

* Biology Department, Brookhaven National Laboratory, Upton, New York 11973, and
Department of Physiology and Biophysics, Health Sciences Center, State University of New York, Stony Brook, New York 11794

Summary. Calcium and other alkaline earth cations change the electrostatic potential adjacent to negatively charged bilayer membranes both by accumulating in the aqueous diffuse double layer adjacent to the membrane and by adsorbing to the phospholipids. The effects of these cations on the electrostatic potential are described adequately by the Gouy-Chapman-Stern theory. We report the results of experiments with ethane-bis-trimethylammonium, a cation that has been termed "dimethonium" or "ethamethonium" in analogy with hexamethonium (hexane-1,6-bis-trimethylammonium) and decamethonium (decane-1,10-bis-trimethylammonium). We examined the effect of dimethonium on the zeta potential of multilamellar vesicles formed from the negative lipid phosphatidylserine (PS) and from 5:1 phosphatidylcholine/phosphatidylserine mixtures in solutions containing 0.1, 0.01 and 0.001 M sodium, cesium, or tetramethylammonium chloride. We also examined the effect of dimethonium on the conductance of planar PS bilayer membranes and the ^{31}P NMR signal from sonicated PS vesicles formed in 0.1 M NaCl. We found no evidence that dimethonium adsorbs specifically to bilayer membranes. All the results, except for those obtained with vesicles of low charge density formed in a solution with a high salt concentration, are consistent with the predictions of the Gouy-Chapman theory. We conclude that dimethonium, which does not have the pharmacological effects of hexamethonium and decamethonium, is a useful divalent cation for physiologists interested in investigating electrostatic potentials adjacent to biological membranes.

Key Words divalent cations · electrostatic potentials · Gouy-Chapman theory · bilayer membranes

Introduction

Calcium exerts a "stabilizing effect" on excitable membranes: a larger depolarization is required to reach the threshold voltage and elicit an action potential when the concentration of calcium bathing a muscle or nerve is increased. Conversely, when the concentration of calcium in the solution bathing a squid axon is reduced, a smaller depolarization is sufficient to reach the threshold voltage, the voltage at which the inward sodium current exceeds the outward current carried by potassium and other ions. Frankenhaeuser and Hodgkin [11] clearly demonstrated that calcium stabilizes squid axons by shifting the conductance-voltage curve along the voltage axis. They also advanced the suggestion, made to them by A.F. Huxley, "that calcium ions may be adsorbed at the outer edge of the membrane and thereby create an electric field inside the membrane which adds to that provided by the resting potential."

Gilbert and Ehrenstein [12], Mozhayeva and Naumov [27] and McLaughlin, Szabo and Eisenman [25] pointed out that calcium could change the surface potential not only by binding to negative charges but also by exerting a "screening" effect in the aqueous diffuse double layer. They used the Gouy-Chapman-Stern theory to describe these effects, and it is now conventional to use this theory to account for the ability of calcium and other divalent cations to both shift the conductance-voltage curves of excitable membranes [4, 10, 15, 16, 31] and change the electrostatic potential adjacent to phospholipid bilayer membranes [18, 21, 24, 30].

The Gouy-Chapman-Stern theory contains two adjustable parameters: the surface potential, ψ, and the intrinsic binding constant of calcium with the membrane, K. Hille and coworkers [8, 15], McLaughlin, Mulrine, Gresalfi, Vaio and McLaughlin [24] and Fohlmeister and Adelman [10] discuss in some detail the difficulties in separating these two parameters. The difficulties arise because the changes in the surface potential are, to a first approximation, a function of the apparent binding constant of calcium with the membrane, K_{app}

$$K_{app} = K \exp(-2F\psi/RT) \quad (1)$$

where R, T, and F have their usual meanings. Thus the shifts in the conductance-voltage curves observed upon addition of calcium could be due to

either a large negative surface potential and a small intrinsic binding constant or a small negative surface potential and a large intrinsic binding constant. For example, the effect of calcium on the potassium channels of squid giant axons can be described by assuming either that $K=0.1\ \text{M}^{-1}$, $\psi=-60$ mV [12] or that $K=30\ \text{M}^{-1}$, $\psi=-15$ mV [10] in the normal bathing solution. As Hille et al. [15] state, "the problem is to find a procedure to identify the shift corresponding to zero surface potential."

We were able to solve this problem for simple phospholipid bilayer membranes by measuring the electrophoretic mobility of multilamellar vesicles. We estimated the intrinsic 1:1 binding constant of calcium with phosphatidylserine [24] and phosphatidylglycerol [18] by determining the concentration of calcium at which a vesicle formed from these lipids reverses charge and becomes positive, $[Ca^{++}]^{rev}$. When the vesicle reverses sign, $\psi=0$ and $K=K_{app}$ (Eq. (1)). The surface potential in the absence of calcium could then be deduced in independent monolayer or bilayer experiments by measuring the change in potential produced by a $[Ca^{++}]=[Ca^{++}]^{rev}$ [2, 9, 24].

Biological membranes, unfortunately, are so heterogeneous that charge reversal measurements would be extremely difficult to interpret. In our opinion, new approaches are required to provide information about the surface potential of excitable membranes. One approach is to find a divalent cation that does not adsorb to phospholipid bilayer membranes. If the Gouy-Chapman theory can describe the ability of this cation to screen the electrostatic potential adjacent to a bilayer, the cation can be used to estimate the surface potential of an excitable membrane by simply comparing the ability of this cation to shift the conductance-voltage curves with the predictions of the Gouy-Chapman theory.

Our search for a suitable divalent cation was guided by our previous experiments with monovalent ions. Tetramethylammonium adsorbs less strongly to negative lipids, such as phosphatidylglycerol (PG) and phosphatidylserine (PS), than do the other monovalent cations we examined [9]: the intrinsic binding constant of tetramethylammonium with PS has been estimated to be 0 and $0.05\ \text{M}^{-1}$ [9, 30]. We concluded that a divalent cation containing two quaternary ammonium moieties would not adsorb strongly to lipids. Hexamethonium is such a cation, but its charges are separated by about 1 nm, the Debye length in a physiological, 0.1 M monovalent salt solution. The large size of hexamethonium decreases the ability

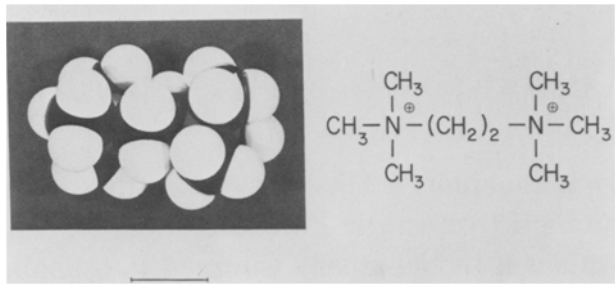

Fig. 1. The structure of dimethonium. *Left:* CPK model. The line represents 0.3 nm. *Right:* chemical formula

of this divalent cation to screen negative charges [2], as predicted theoretically [7]. We report measurements with dimethonium (Fig. 1), a two carbon analog of hexamethonium. Our zeta potential, conductance and NMR measurements are consistent with the postulate that dimethonium does not adsorb to lipids but exerts only a screening effect on the electrostatic potential adjacent to negatively charged bilayer membranes. The charged quaternary nitrogens in dimethonium are separated by about 0.3 nm. Although dimethonium can hardly be considered a point charge, it is smaller than the Debye length in a physiological solution and the screening effect can be adequately described, under most conditions, by the Gouy-Chapman theory of the diffuse double layer.

Materials and Methods

Synthesis of Dimethonium

Dimethonium [32] was prepared by reacting N,N′-tetramethylethylenediamine, which was redistilled immediately before use, with methyl bromide in acetonitrile, followed by recrystallizations from isobutanol/methanol. The chemical structure and purity (<0.3% impurities) of the dimethonium was confirmed by proton NMR at 360 MHz.

Electrophoretic Mobility Measurements

Multilamellar vesicles were prepared following the method of Bangham et al. [3] from egg phosphatidylcholine, bovine brain phosphatidylserine (PC, PS; Avanti Biochemicals, Birmingham, AL) or mixtures of these two lipids. Water was purified with a Super-Q system (Millipore Corp., Bedford, MA), then double distilled in a quartz still and stored in quartz flasks. Tetramethylammonium chloride was recrystallized twice before use; the concentrations of all electrolytes in the stock solutions were checked by measuring the conductivity. The 0.1, 0.01, and 0.001 M monovalent salt solutions were buffered to pH 7.5 with 1.0, 0.1 and 0.01 mM 3-(N-Morpholino)-Propanesulfonic acid (MOPS), respectively. Electrophoretic mobility measurements were made at 25 °C with Rank Bros. Mark I microelectrophoresis machines (Bottisham, Cambridge, UK). All measurements were made at the stationary layer [14] and the current was monitored to ensure that electrode polarization did

not occur. The main source of error was the settling of vesicles, which changes the position of the stationary layer in the electrophoresis tube. The zeta potential, ζ, the electrostatic potential at the hydrodynamic plane of shear, was calculated from the electrophoretic mobility, u, using the Helmholtz-Smoluchowski equation:

$$\zeta = u\eta/\varepsilon_r\varepsilon_o \quad (2)$$

where η is the viscosity, ε_r is the dielectric constant and ε_o is the permittivity of free space. When the salt concentration was low and the surface potential was large, measurements were made on large vesicles (diameter > 10 μm) to circumvent the relaxation effect [9, 36].

In order to compare the zeta potential results with the predictions of the Gouy-Chapman-Stern theory of the diffuse double layer, we need to know the area per lipid head group and the distance of the plane of shear from the membrane. We assume that both PC and PS occupy 0.7 nm^2 in a bilayer membrane, that the plane of shear is 0.2 nm from the surface in 0.1 M monovalent salt solutions [2, 9], 0.4 nm in 0.01 M solutions, and 1 nm from the surface in the 0.001 M solutions (Appendix A).

Conductance Measurements

The planar bilayer membranes were formed from a 10 mg/ml PS/decane solution. The aqueous phases contained either 0.1 M NaCl, 0.01 M KCl, 0.001 M MOPS, pH 7.5, 1 μM nonactin or 0.1 M NaCl, 0.01 M MOPS, pH 7.5, 1 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The initial conductance of the PS membrane, G^0, was measured, dimethonium was added to the aqueous phase, and the conductance was measured again, G. The change in electrostatic potential within the interior of the membrane, $\Delta\psi$, was calculated from

$$\Delta\psi = \pm(RT/F)\ln(G/G^o) \quad (3)$$

where the negative sign is taken for the nonactin-induced conductance and the positive sign is taken for the FCCP-induced conductance. We discuss elsewhere the evidence that Eq. (2) can be used to calculate the change in surface potential from the nonactin [25, 26, 33] and FCCP [5] conductances.

^{31}P NMR Measurements

Low concentrations of cobalt broaden the ^{31}P NMR linewidth of sonicated phospholipid vesicles [18, 20]. The broadening is proportional to the number of phosphodiester groups bound in inner sphere complexes with cobalt, which is proportional to the free concentration of cobalt in the aqueous phase adjacent to the phosphate group. This free concentration is proportional to the Boltzmann factor, $\exp(-2F\psi_p/RT)$, where ψ_P is the electrostatic potential at the phosphodiester group. The potential in the bulk aqueous phase is defined to be zero. Changes in the ^{31}P NMR signal can be used to estimate changes in the potential at the phosphodiester group upon addition of dimethonium because the cobalt concentration used in these studies (8 μM) affects neither ψ_P [20] nor the zeta potential [24] of PS vesicles in 0.1 M NaCl. If we assume that the number of cobalt binding sites remains constant upon addition of the divalent cations calcium or dimethonium, the ratio of the ^{31}P NMR linewidth in the presence, $1/T_{2P}$, and absence, $1/T^o_{2P}$ of these divalent cations is given by the expression:

$$(1/T_{2P})/(1/T^o_{2P}) = \exp(-2F\Delta\psi_P/RT) \quad (4)$$

where $\Delta\psi_P$ is the change in the potential at the phosphodiester group upon the addition of divalent cations (*see* Appendix B).

The observed linewidths were corrected by subtracting the natural linewidth. The free concentrations of cobalt, calcium, and dimethonium were established by passing the sonicated PS sample through a sephadex column. Freshly sonicated samples were used for each experimental point shown in Figs. 11 and 12. All experiments were performed at 25 °C.

Results

Zeta Potential Measurements

Measurements in 0.1 M NaCl

Figure 2 illustrates the effect of the divalent cation dimethonium on the zeta potential of PS (triangles) and 5:1 PC/PS (squares) vesicles formed in 0.1 M NaCl. The effect of dimethonium on the PS vesicles is described well by the theoretical screening curve, the solid line. Thus it is not necessary to invoke any specific adsorption of dimethonium to PS to account for the effect of this cation on the electrostatic potential adjacent to the membrane.

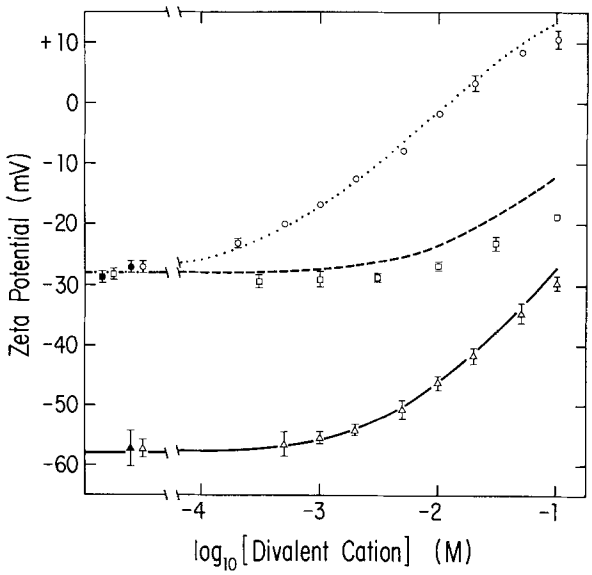

Fig. 2. The effect of divalent cations on the zeta potentials of vesicles formed in 0.1 M NaCl. Triangles: the effect of dimethonium on vesicles formed from the negative lipid phosphatidylserine, PS. Squares: the effect of dimethonium on vesicles formed from 5:1 mixtures of the zwitterionic lipid phosphatidylcholine (PC) with PS. Circles: the effect of calcium on 5:1 PC/PS vesicles. The vertical lines in this and the following figures indicate the standard deviations of measurements on at least 20 different vesicles in two different experiments. The filled symbols in this and the following figures indicate the zeta potentials when the solutions also contained trace concentrations (10^{-4} or 10^{-5} M) of EDTA. The solid and dashed curves illustrate the predictions of the Gouy-Chapman-Stern theory when the association constant of dimethonium with the membrane is assumed to be zero and the intrinsic Na−PS association constant is assumed to be 1 M^{-1}

However, the results obtained with dimethonium and the less negative 5:1 PC/PS vesicles can only be qualitatively described by the theoretical screening curve. Dimethonium has a smaller effect on the zeta potential of these vesicles than predicted by the screening curve (dashed line). On the other hand, calcium (circles) has a much larger effect on the zeta potential of 5:1 PC/PS vesicles than predicted by the screening curve. When $[Ca^{++}]$ is greater than about 0.01 M, the vesicles reverse sign and become positive, a clear indication that calcium adsorbs specifically to the vesicles. The Gouy-Chapman-Stern theory, which combines the Gouy-Chapman model of the aqueous diffuse double layer with the Langmuir adsorption isotherm and the Boltzmann relation, provides the simplest description of these results. The theory is discussed in detail elsewhere [24], and the equations will not be repeated here. The prediction of the theory is illustrated by the dotted line in Fig. 2. The theoretical curves illustrated in this report were all drawn assuming that the intrinsic association constant of sodium with the negative lipid PS is 1 M^{-1}, a value consistent with the estimates of 0.6–1.0 M^{-1} in the literature [9, 17, 28], that the 1:1 association constant of calcium with PS is 12 M^{-1} [24] and that the plane of shear is 0.2 nm from the surface in a decimolar monovalent salt solution [2, 9]. In order to provide a good fit to the calcium data the dotted curve in Fig. 2 was drawn assuming that the 1:1 association constant of calcium with the zwitterionic lipid PC is 6 M^{-1}; this number agrees, within a factor of two, with the value determined from experiments with PC vesicles [24]. We ignore any possible adsorption of anions to the vesicles [13].

Measurements in 0.01 M NaCl

When [NaCl] is lowered from 0.1 (Fig. 2) to 0.01 M (Fig. 3) the zeta potential of the negatively charged vesicles become more negative[1] and divalent cations, such as dimethonium and calcium, affect the electrostatic potential at lower bulk aqueous concentrations. The theory of the diffuse double layer predicts both of these effects. The solid and dashed curves in Fig. 3 illustrate the predicted screening curves for PS and 5:1 PC/PS vesicles, respectively. That is, the curves are the predictions of the Gouy-Chapman-Stern theory if the association constant of dimethonium with the membrane is zero and the intrinsic Na–PS association constant is 1 M. These screening curves describe the experimental data obtained with dimethonium. In both 0.01 M NaCl (Fig. 3) and 0.1 M NaCl (Fig. 2), calcium is much more effective than dimethonium in reducing the zeta potential of PC/PS vesicles. In both cases (Figs. 2 and 3) the PC/PS vesicle reverse sign when $[Ca^{++}] \simeq 0.01$ M, indicating that calcium is adsorbing specifically to the membranes. The calcium data in Fig. 3 are described adequately by the Gouy-Chapman-Stern theory (dotted line).[2]

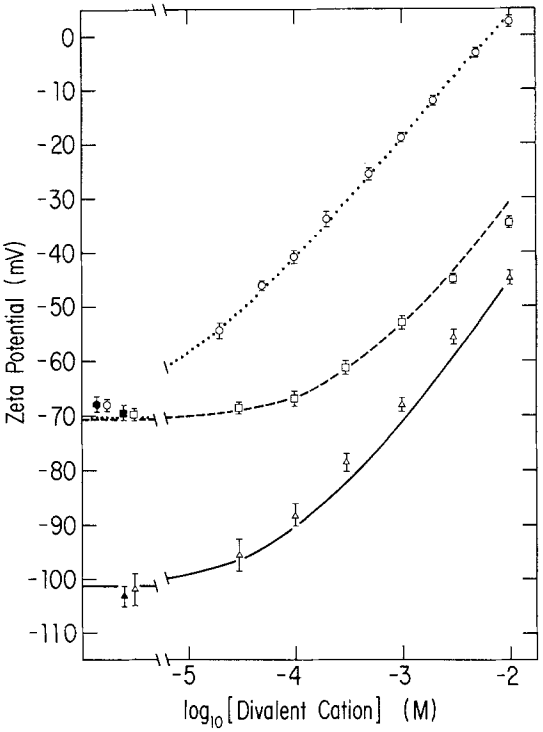

Fig. 3. The effect of divalent cations on the zeta potentials of vesicles formed in 0.01 M NaCl. Triangles: the effect of dimethonium on PS vesicles. Squares: the effect of dimethonium on 5:1 PC/PS vesicles. Circles: the effect of calcium on 5:1 PC/PS vesicles

[1] When the monovalent salt concentration is reduced to values below 0.1 M, the magnitude of the zeta potential does not increase as much as predicted by the Gouy-Chapman-Stern theory if it is assumed that the hydrodynamic plane of shear remains a constant distance from the surface. As discussed in more detail in Appendix A, either the equilibrium double layer theory is inadequate or the hydrodynamic plane of shear moves out from the surface as the salt concentration decreases. It is difficult to distinguish between these two possibilities with zeta potential measurements alone, and we do not attempt to do so here. The distance of the plane of shear from the membrane, which was shown experimentally to be 0.2 nm from the surface in a 0.1 M monovalent salt solution [2, 9], was assumed to be 0.4 nm from the surface in the 0.01 M and 1 nm from the surface in the 0.001 M monovalent salt solutions.

[2] We assumed that the association constant of calcium with PC was 6 M^{-1} in the 0.1 M NaCl solution (Fig. 2). It was necessary to use a Ca–PC association constant of 12 M^{-1} to provide a good fit to the data obtained in 0.01 (Fig. 3) and 0.001 (Fig. 4) M NaCl. This apparent increase in the Ca–PC association constant is probably not real. The theory we use is highly oversimplified; it ignores, for example, the increase in the activity coefficient of calcium that will occur when the monovalent salt concentration is reduced, a phenomenon that at least partially accounts for the apparent change in the association constant.

Measurements in 0.001 M NaCl

Figure 4 illustrates the data obtained with 5:1 PC/PS vesicles when [NaCl] = 0.001 M. The two trends seen in Fig. 3 upon lowering [NaCl] continue in Fig. 4: the magnitude of the potential increases and divalent cations become more effective in reducing the potential. The magnitude of the zeta potential of the 5:1 PC/PS vesicles increases from a value of about −70 mV in 0.01 M NaCl (Fig. 3) to a value of about −110 mV in 0.001 M NaCl (Fig. 4). Divalent cations are more effective in reducing the magnitude of the surface potential at this lower [NaCl] mainly because the potential is larger. The dimethonium data obtained at 0.001 M NaCl are described well by the simple "screening" theory (dashed curve). The data obtained with calcium are described by the Gouy-Chapman-Stern theory (dotted curve) with the same values for the Na−PS and Ca−PS association constants that were used to fit the data obtained in 0.01 M NaCl. We cannot measure the electrostatic potentials of PS vesicles at this low salt concentration for technical reasons.[3]

The results presented in Figs. 2, 3 and 4 can be summarized very simply. With the exception of vesicles formed from 5:1 PC/PS mixtures in 0.1 M NaCl, the Gouy-Chapman-Stern theory accounts for the effects of both calcium and dimethonium on the zeta potential of phospholipid vesicles. Dimethonium appears to exert only a screening effect: there is no need to postulate any specific adsorption of this cation to the membranes.

Measurements in CsCl solutions

The interpretation of the results presented in Figs. 2–4 is complicated by the adsorption of sodium to the membrane [9]. If dimethonium acts only to screen the charges on the membrane, the experimental results obtained when the membranes are formed in a solution containing a monovalent cation that does not adsorb to the membrane should also agree with the predictions of the screening theory. Cesium binds less strongly to negative lipids such as phosphatidylserine and phosphatidylglycerol than do the other alkali metal cations [9]. The results illustrated in Figs. 5, 6 and 7 were obtained under conditions similar to those in Figs. 2, 3 and 4, except that the solutions contained Cs instead of Na. The zeta potentials of vesicles formed in CsCl are more negative than the zeta potentials of vesicles formed in NaCl. For example, the zeta potential of a PS vesicle formed in 0.1 M NaCl is −58 mV (Fig. 2), whereas the zeta potential of a PS vesicle formed in 0.1 M CsCl is −75 mV (Fig. 5). If the association constant of

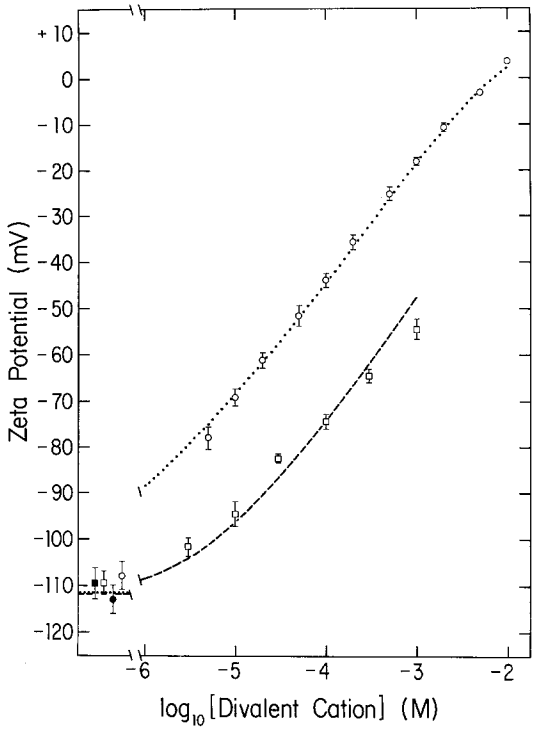

Fig. 4. The effect of divalent cations on the zeta potentials of vesicles formed in 0.001 M NaCl. Squares: the effect of dimethonium on 5:1 PC/PS vesicles. Circles: the effect of calcium on 5:1 PC/PS vesicles

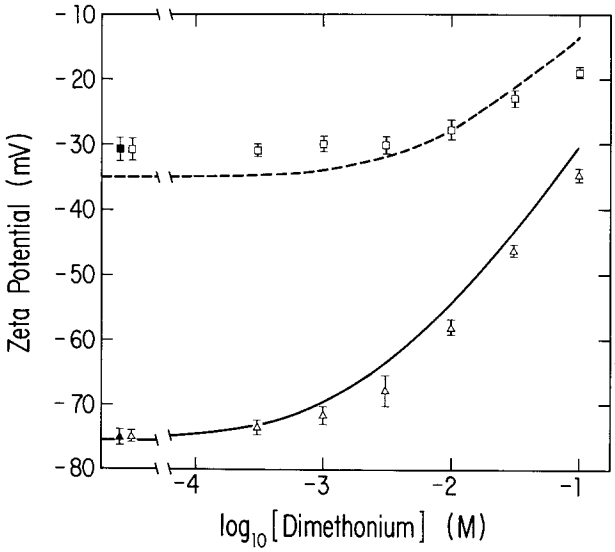

Fig. 5. The effect of dimethonium on the zeta potentials of vesicles formed in 0.1 M CsCl. Triangles: PS vesicles. Squares: 5:1 PC/PS vesicles. The curves in this and the following two figures illustrate the predictions of the Gouy-Chapman-Stern theory with the association constant of the Cs−PS complex assumed to be 0.1 M^{-1}, and the association constant of dimethonium with the membrane assumed to be zero

[3] In order to circumvent the "relaxation" effect [9, 36] in the 0.001 M NaCl solution it is necessary to make measurements on large (diameter greater than 10 μm) vesicles. We were not able to form such large vesicles from PS in 0.001 M NaCl, CsCl or TMACl.

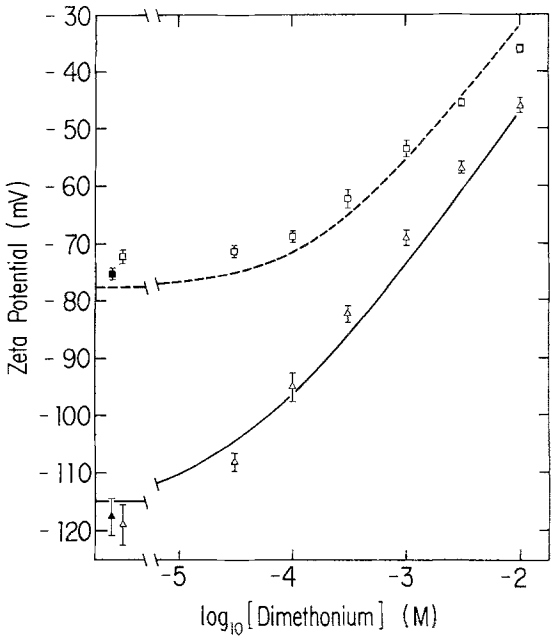

Fig. 6. The effect of dimethonium on the zeta potentials of vesicles formed in 0.01 M CsCl. Triangles: PS vesicles. Squares: 5:1 PC/PS vesicles. The curves illustrate the theoretical predictions of the screening theory

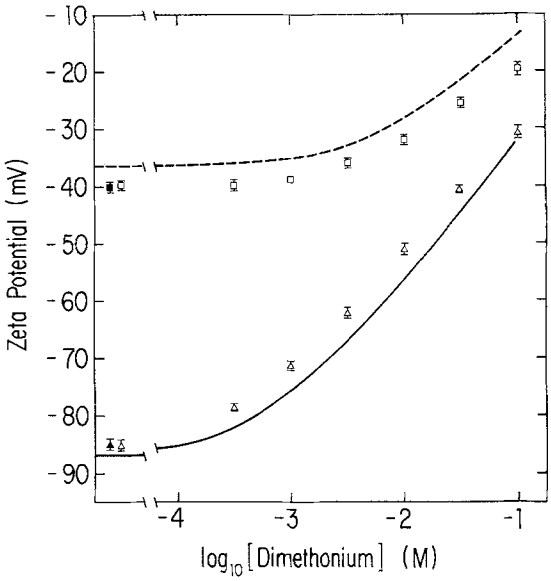

Fig. 8. The effect of dimethonium on the zeta potentials of vesicles formed in 0.1 M TMACl. Triangles: PS vesicles. Squares: 5:1 PC/PS vesicles. The curves illustrate the theoretical predictions of the Gouy-Chapman screening theory; it is assumed that neither TMA nor dimethonium bind to the membrane

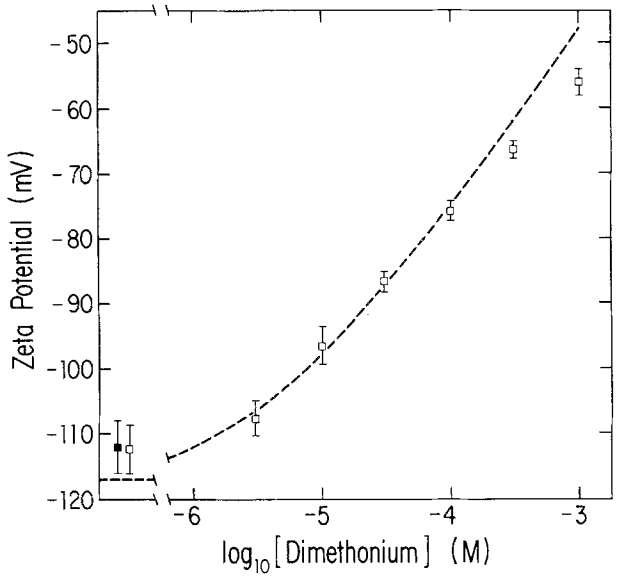

Fig. 7. The effect of dimethonium on the zeta potentials of 5:1 PC/PS vesicles formed in 0.001 M CsCl. The curve illustrates the predictions of the screening theory

Cs with PS is assumed to be 0.1 M^{-1}, a reasonable fit to the initial data points is obtained using the Gouy-Chapman-Stern theory. The effect of dimethonium on the zeta potential can be described adequately by the Gouy-Chapman-Stern theory under most conditions if it is assumed that dimethonium does not adsorb to the membranes (Figs. 5, 6 and 7). The exception is the result obtained with the 5:1 PC/PS vesicles in 0.1 M CsCl.

Measurements in Tetramethylammonium Chloride (TMACl) Solutions

Figures 8, 9 and 10 illustrate the results obtained when vesicles are formed in solutions containing TMACl. The zeta potentials are more negative when the vesicles are formed in TMA solutions than when they are formed in solutions containing either Cs or Na. For example, the zeta potentials of PS vesicles in 0.1 M NaCl, CsCl and TMACl solutions are −58, −75 and −85 mV, respectively. Presumably TMA binds less strongly to PS than the alkali metal cations. The theoretical screening curves in Figs. 8, 9 and 10 were drawn assuming that neither dimethonium nor TMA adsorb to the membranes;[4] we stress that these theoretical curves do not have any adjustable parameters. The agreement between the theoretical curves and experimental points, although not perfect, is considered to be adequate in all cases.

[4] There is some evidence from NMR experiments [1] and monolayer surface potential measurements [30] that TMA does bind weakly to phospholipid bilayer membranes.

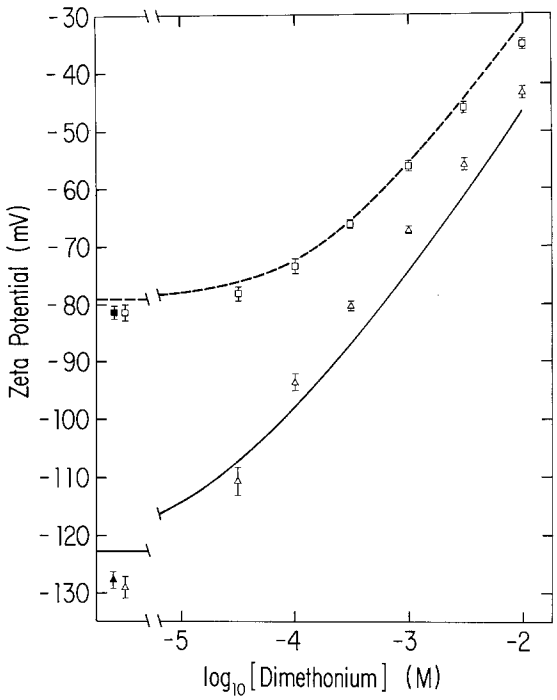

Fig. 9. The effect of dimethonium on the zeta potentials of vesicles formed in 0.01 M TMACl. Triangles: PS vesicles. Squares: 5:1 PC/PS vesicles. The curves illustrate the theoretical predictions of the Gouy-Chapman screening theory

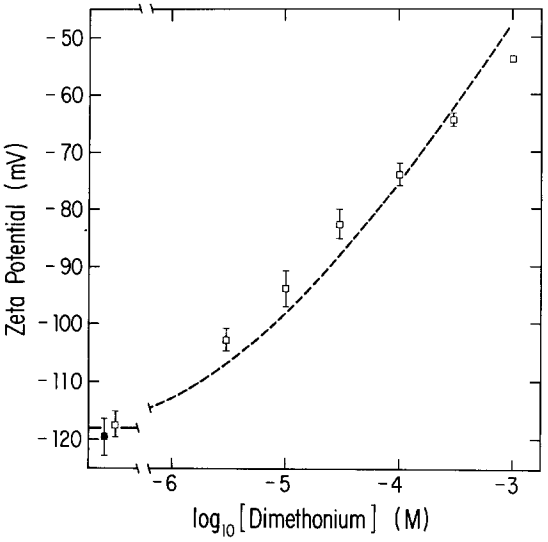

Fig. 10. The effect of dimethonium on the zeta potentials of 5:1 PC/PS vesicles formed in 0.001 M TMACl. The curve illustrates the prediction of the Gouy-Chapman screening theory

Control Experiments with PC Vesicles

If our claim that dimethonium does not adsorb to phospholipids is correct then this cation should not produce a positive zeta potential when added to a solution containing PC vesicles. When 0.1 M dimethonium bromide is added to a 0.1 M NaCl solution containing PC vesicles it produces a small negative zeta potential (-6 ± 1 mV, $n=40$) on the initially neutral ($\zeta=0\pm1$ mV, $n=20$) vesicles. This can be accounted for partially by the adsorption of bromide ions to the membrane (the addition of 0.2 M NaBr produces a zeta potential of -2 ± 1 mV, $n=30$, on PC vesicles formed in 0.1 M NaCl) and partially by the finite size of the dimethonium ion [7, 35].

Conductance Measurements

Dimethonium decreases the nonactin-induced conductance of a PS membrane formed in a solution containing 0.1 M NaCl. A few control experiments indicate that it produces a symmetrical increase in the FCCP-induced conductance. The changes in the electrostatic potential within the membrane were calculated from the nonactin data using Eq. (2); they were 11 ± 3 mV ($n=3$) when [dimethonium bromide] = 24 mM and 22 ± 1 mV ($n=3$) when [dimethonium bromide] = 68 mM. The Gouy-Chapman-Stern theory predicts that these dimethonium concentrations produce changes in the surface potential of 16 and 23 mV if the Na−PS association constant is 1 M^{-1} and the dimethonium-PS association constant is zero. The simple screening theory thus describes both the zeta potential (Fig. 2) and the nonactin conductance results. The nonactin-induced conductance, in contrast to the electrophoretic mobility, responds to changes in the dipole potential at the membrane-solution interface [21]. If dimethonium is to be useful to biologists it should not, and it does not, change the dipole potential of a bilayer membrane.

NMR Measurements

We used ^{31}P NMR-measurements to determine the change in the electrostatic potential at the phosphodiester group of PS upon addition of a divalent cation. The data points in Fig. 11*A* illustrate the effect of calcium on the ^{31}P NMR linewidth of sonicated PS vesicles when the solution contained 0.1 M NaCl and a free cobalt concentration of 8 μM. The data points in Fig. 11*B* illustrate the change in the electrostatic potential at the phosphodiester group, $\Delta\psi_P$, produced by calcium. $\Delta\psi_P$ was calculated in two different ways. The lower value for each pair of points in Fig. 11*B* was estimated from the corresponding point in Fig. 11*A* using Eq. (4), which follows from the assumption that neither sodium nor calcium compete with cobalt for the binding site. The upper value for each pair of points in Fig. 11*B* was estimated using the combination of Eqs. (B1), (B2) and (B3). In mak-

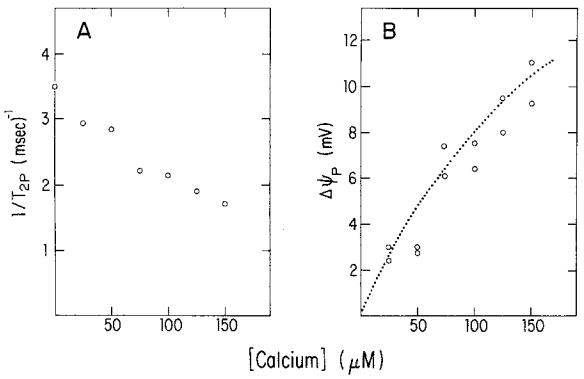

Fig. 11. (A.) The effect of calcium on the ^{31}P NMR linewidth, $1/T_{2P}$, of PS molecules in the outer monolayer of sonicated vesicles in 0.1 M NaCl. The solutions also contained 8 μM free cobalt and 1 mM MES buffered to pH 5.0 at 25 °C. (*B*): The change in the potential at the phosphodiester group, $\Delta\psi_P$, produced by calcium. The lower set of circles was determined from the corresponding points in *A* by assuming that the number of binding sites for cobalt remained constant on addition of calcium. The upper set of circles was determined by assuming that sodium and calcium ions compete with cobalt for the binding site. The curve is the change in the surface potential predicted by the Gouy-Chapman-Stern theory.

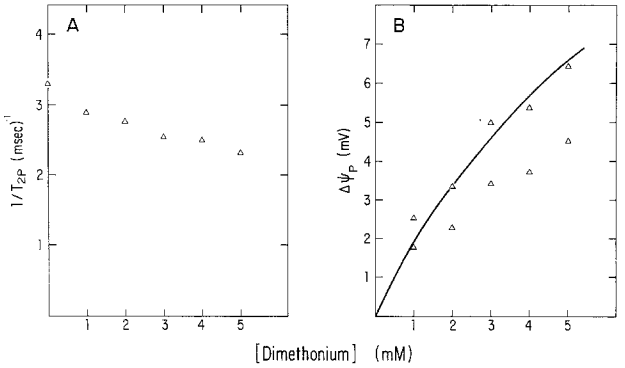

Fig. 12. (*A*): The effect of dimethonium on the ^{31}P NMR linewidth, $1/T_{2P}$, of PS molecules in the outer monolayer of sonicated vesicles. The solutions contained 0.1 M NaCl, 8 μM free cobalt and 1 mM MES buffered to pH 5.0 at 25 °C. (*B*): The change in the potential at the phosphodiester group, $\Delta\psi_P$, produced by dimethonium. The lower set of triangles was determined from the corresponding points in *A* by assuming that the number of binding sites for cobalt does not change on addition of dimethonium. The upper set of triangles was determined by assuming that sodium ions compete with cobalt for the binding site. The curve is the change in the surface potential predicted by the the Gouy-Chapman-Stern theory for a point divalent cation that does not adsorb to the membrane

ing this calculation we assume that the competition between sodium, calcium and cobalt for the binding site can be described by the Langmuir adsorption isotherm (Appendix B). The curve in Fig. 11 *B* illustrates the prediction of the Gouy-Chapman-Stern theory for the change in the surface potential, assuming that the 1:1 association constant of the Na−PS complex is 1 M^{-1} and that the 1:1 intrinsic association constant of the Ca−PS complex is 12 M^{-1} [24]. The theory describes adequately the effect of calcium on the potential at the phosphate group.

The data points in Fig. 12 *A* illustrate the effect of dimethonium on the ^{31}P NMR linewidth of sonicated PS vesicles formed in a solution containing 0.1 M NaCl and 8 μM free cobalt. The data points in Fig. 12 *B* illustrate the change in the electrostatic potential at the phosphodiester group, $\Delta\psi_P$, which was calculated in the same two ways as in Fig. 11 *B*. Dimethonium is about 20 times less effective than calcium in reducing the potential at the phosphate group. The curve in Fig. 12 *B* illustrates the change in the surface potential predicted by the Gouy-Chapman-Stern theory, assuming that the Na−PS association constant is 1 M^{-1} and that dimethonium does not adsorb to the membrane. This "screening" curve for a hypothetical point divalent cation describes adequately the effect of dimethonium on the potential at the phosphate group. In summary, the NMR experiments agree very well with the zeta potential and conductance results, and are consistent with the postulate that dimethonium does not adsorb to PS membranes but exerts only a screening effect on the potential. Furthermore, this screening effect can be described reasonably well by the theory of the diffuse double layer.

Discussion

Our main conclusion is that the simple Gouy-Chapman-Stern theory describes the effects of small divalent cations on the electrostatic potential adjacent to phospholipid bilayer membranes surprisingly well. The theory describes both the effects of calcium, which binds to the membrane as well as screening the surface charges, and the effects of dimethonium, which exerts only a screening effect.

We consider first the slopes of the surface and zeta potential *vs.* [divalent cation] curves. The Gouy-Chapman-Stern theory predicts that the maximum slope should be 27 mV/decade for both adsorbing and nonadsorbing divalent cations. This prediction is consistent with the experimental results we obtained with both calcium and dimethonium (Figs. 1–10). The prediction is also consistent with the results obtained with calcium using many other techniques [2, 18, 24, 25, 26, 30]. A slope greater than the theoretically predicted value is conventionally taken to be evidence for a discreteness-of-charge effect, which is assumed to be negligible in the Gouy-Chapman-Stern theory [23]. The

zeta potential results presented here are consistent with the assumption that discreteness-of-charge effects are not of great importance in the interaction of small divalent cations with bilayer membranes. This conclusion agrees with the NMR results reported here and elsewhere [18] which show that the Gouy-Chapman-Stern theory describes the effect of calcium on the micropotential at the phosphodiester group.

The Gouy-Chapman-Stern theory describes not only the slope of the curves, but also the ability of divalent cations to change the electrostatic potential under a variety of experimental conditions. We consider the results that have been obtained with calcium when the bilayer membranes contain the negative lipid PS. Zeta potential measurements on multilamellar vesicles (Figs. 1–10, this report; [24]), direct surface potential measurements on PS monolayers [30], nonactin-conductance and capacitance measurements on planar PS bilayers [2] as well as calcium electrode [24] and NMR (Fig. 11) measurements on sonicated PS vesicles are all consistent with the predictions of the Gouy-Chapman-Stern theory if the 1:1 intrinsic association constant of calcium with PS is taken to be of order 10 M^{-1}. Thus calcium changes the potential at the membrane-solution interface by both changing the charge density and screening the surface charges.

Dimethonium, on the other hand, appears to exert only a screening effect on phospholipid bilayer membranes. The effect of dimethonium on the zeta potential (Figs. 1–10), the potential at the phosphodiester group (Fig. 12) and the potential within the membrane (conductance results) can be described quite well by the Gouy-Chapman-Stern theory if it is assumed that this cation does not adsorb to the membranes. Significant deviations were observed only when membranes with a low charge density were formed in solutions containing a high concentration of monovalent salt: dimethonium then had less than the predicted screening effect (Fig. 2). All our results, therefore, are consiistent with the postulate that dimethonium does not adsorb to phospholipid bilayer membranes.

It is interesting to compare our dimethonium results with the predictions of modern electrostatic theories of the diffuse double layer. Both the modified Poisson-Boltzmann approach of Bhuiyan, Outhwaite and Levine [6] and the Monte Carlo calculations of Torrie and Valleau [35] predict quite different results than the Gouy-Chapman-Stern theory. Torrie and Valleau assumed that the diameter of the divalent cation was 0.425 nm, which is smaller than the diameter of the dimethonium molecule (Fig. 1). The Monte Carlo calculations suggest that 0.05 M divalent cation will reduce the potential, at distances 0.2 nm and further from a surface with a charge density similar to that of a PS membrane, to values much smaller than those predicted by the Gouy-Chapman theory (*see* Fig. 5 of [35]). Our zeta potential results, however, are consistent with predictions of the Gouy-Chapman theory. Thus the simple, classical theory provides a better description of the experimental results than do the complicated, modern theories. This finding should please both theoreticians, who must now explain why the classical theory works so well, and experimentalists, who can continue to use the simple theory to describe their results.

There is some controversy about the surface potentials adjacent to electrically excitable channels in biological membranes [10, 16] and experiments with dimethonium may provide valuable information about these potentials. In the past, the ability of calcium to shift the conductance-voltage curves has been used to estimate the surface potential. The intrinsic association constant of calcium with the membrane was generally assumed to be of order 0.1 M^{-1} [4, 12, 15]. However, all biological membranes contain lipids in the form of a bilayer and the intrinsic association constant of calcium with both negative and zwitterionic lipids is 1–2 orders of magnitude greater than 0.1 M^{-1} [22]. It seems likely to us that the intrinsic association constant of calcium with biological membranes has been underestimated, and that the magnitude of the surface potential has been overestimated. For example, Hille and coworkers [15] estimated that the surface potential adjacent to the sodium channels of a myelinated frog nerve in Ringer solution was between −60 and −90 mV. These values bracket the surface potential of a PS membrane in 0.1 M NaCl, which is about −80 mV [9]. If the magnitude of the surface potential adjacent to the sodium channels of frog myelinated nerves [15] and frog skeletal muscle [8] is actually this high, then the addition of 10 mM dimethonium to the bathing solution should produce a shift in the conductance-voltage curve of about 10 mV (*see* Figs. 2 and 12). It does not: 20 mM dimethonium produces no significant shift in the conductance-voltage curves of sodium channels in frog skeletal muscle [8]. This result suggests, but does not prove [8], that the surface potential adjacent to the sodium channel in skeletal muscle is less than −40 mV in magnitude.[5] It also illustrates how dimethonium may be useful to physiologists interested in the sur-

[5] For example, in the absence of divalent cations the zeta potential of 5:1 PC/PS vesicles in 0.1 M NaCl is about −30 mV and the surface potential is about −40 mV; 10 mM dimethonium has little effect on the zeta potential of such vesicles (Fig. 2).

face potential adjacent to an electrically excitable channel in biological membranes.

We thank Chris Miller for advice about the synthesis of dimethonium. This work was supported by NIH grant GM24971 to S.McL. and A.McL., NSF grant PCM 82-00991 to S. McL., and Council for Tobacco Research grant 1493 to A. McL.

References

1. Akutsu, H., Seelig, J. 1981. Interaction of metal ions with phosphatidylcholine bilayer membranes. *Biochemistry* **20**:7366–7373
2. Alvarez, O., Brodwick, M., Latorre, R., McLaughlin, A., McLaughlin, S., Szabo, G. 1983. Large divalent cations and electrostatic potentials adjacent to membranes: Experimental results with hexamethonium. *Biophys. J. (in press)*
3. Bangham, A.D., Hill, M.W., Miller, N.G.A. 1974. Preparation and use of liposomes as models of biological membranes. *Methods Membr. Biol.* **1**:1–68
4. Begenisich, T. 1975. Magnitude and location of surface charges on *Myxicola* giant axons. *J. Gen. Physiol.* **66**:47–65
5. Benz, R., McLaughlin, S. 1983. The molecular mechanism of action of the proton ionophore FCCP. *Biophys. J.* **41**:381–398
6. Bhuiyan, L.B., Outhwaite, C.W., Levine, S. 1981. Numerical solution of a modified Poisson-Boltzmann equation for 1:2 and 2:1 electrolytes in the diffuse layer. *Mol. Phys.* **42**:1271–1290
7. Carnie, S., McLaughlin, S. 1983. Large divalent cations and electrostatic potentials adjacent to membranes: A theoretical calculation. *Biophys. J. (in press)*
8. Dani, J., Sanchez, J.A., Hille, B. 1983. Lyotropic anions: Na channel gating and Ca electrode response. *J. Gen. Physiol.* **81**:255–281
9. Eisenberg, M., Gresalfi, T., Riccio, T., McLaughlin, S. 1979. Adsorption of monovalent cations to bilayer membranes containing negative phospholipids. *Biochemistry* **18**:5213–5223
10. Fohlmeister, J.F., Adelman, W.J., Jr. 1982. Periaxonal surface calcium binding and distribution of charge on the faces of squid axon potassium channel molecules. *J. Membrane Biol.* **70**:115–123
11. Frankenhaeuser, B., Hodgkin, A.L. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol. (London)* **137**:218–244
12. Gilbert, D., Ehrenstein, G. 1969. Effect of divalent cations on potassium conductance of squid axons: Determination of surface charge. *Biophys. J.* **9**:447–463
13. Grasdalen, H., Eriksson, L.E.G., Westman, J., Ehrenberg, A. 1977. Surface potential effects on metal ion binding to phosphatidylcholine membranes. *Biochim. Biophys. Acta* **469**:151–162
14. Henry, D.C. 1938. A source of error in micro-cataphoretic measurements with a cylindrical-bore cell. *J. Chem. Soc. (London)* 997–999
15. Hille, B., Woodhull, A.M., Shapiro, B.I. 1975. Negative surface charge near sodium channels of nerve: Divalent ions, monovalent ions, and pH. *Phil. Trans. R. Soc. London B.* **270**:301–318
16. Kostyuk, P.G., Mironov, S.L., Doroshenko, P.A., Ponomarev, V.N. 1982. Surface charges on the outer side of mollusc neuron membrane. *J. Membrane Biol.* **70**:171–179
17. Kurland, R., Newton, C., Nir, S., Papahadjopoulos, D. 1979. Specificity of Na^+ binding to phosphatidylserine vesicles from a ^{23}Na NMR relaxation study. *Biochim. Biophys. Acta* **551**:137–147
18. Lau, A., McLaughlin, A., McLaughlin, S. 1981. The adsorption of divalent cations to phosphatidylglycerol bilayer membranes. *Biochim. Biophys. Acta* **645**:279–292
19. MacDonald, R.C., Bangham, A.D. 1972. Comparison of double layer potentials in lipid monolayers and lipid bilayer membranes. *J. Membrane Biol.* **7**:29–53
20. McLaughlin, A. 1982. Phosphorous-31 and carbon-13 nuclear magnetic resonance studies of divalent cation binding to phosphotidylserine membranes: Use of cobalt as a paramagnetic probe. *Biochemistry* **21**:4879–4885
21. McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interfaces. *Curr. Top. Membr. Transp.* **9**:71–144
22. McLaughlin, S. 1982. Divalent cations, electrostatic potentials, bilayer membranes. *In:* Membranes and Transport. A. Martonosi, editor. Vol. 1, pp. 51–55. Plenum Press, New York
23. McLaughlin, S. 1983. Experimental tests of the assumptions inherent in the Gouy-Chapman-Stern theory of the aqueous diffuse double layer. *In:* Physical Chemistry of Transmembrane Ion Movements. G. Spach, editor. Elsevier, Amsterdam
24. McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G., McLaughlin, A. 1981. Adsorption of divalent cations to bilayer membranes containing phosphatidylserine. *J. Gen. Physiol.* **77**:445–473
25. McLaughlin, S.G.A., Szabo, G., Eisenman, G. 1971. Divalent ions and the surface potential of charged phospholipid membranes. *J. Gen. Physiol.* **58**:667–687
26. McLaughlin, S.G.A., Szabo, G., Eisenman, G., Ciani, S. 1970. Surface charge and the conductance of phospholipid membranes. *Proc. Natl. Acad. Sci. USA* **67**:1268–1275
27. Mozhayeva, G.N., Naumov, A.P. 1970. Effect of surface charge on the steady-state potassium conductance of nodal membrane. *Nature (London)* **228**:164–165
28. Nir, S., Newton, C., Papahadjopoulos, D. 1978. Binding of cations to phosphatidylserine vesicles. *Bioelectrochem. Bioenerg.* **5**:116–133
29. Ohki, S. 1981. Membrane potentials, surface potential, and ionic permeabilities. *Physiol. Chem. Phys.* **13**:195–210
30. Ohki, S., Kurland, R. 1981. Surface potential of phosphatidylserine monolayers: II. Divalent and monovalent binding. *Biochim. Biophys. Acta* **645**:170–176
31. Schauf, C.L. 1975. The interactions of calcium with *Myxicola* giant axons and a description in terms of a simple surface charge model. *J. Physiol. (London)* **248**:613–624
32. Shindo, H., Takahashi, I., Nakajima, E. 1971. Autoradiographic studies on the distribution of quaternary ammonium compounds. II. Distribution of ^{14}C-labeled decamethonium, hexamethonium and dimethonium in mice. *Chem. Pharm. Bull.* **19**:1876–1885
33. Szabo, G., Eisenman, G., McLaughlin, S.G.A., Krasne, S. 1972. Ionic probes of membrane structure. *Ann. N.Y. Acad. Sci.* **195**:273–290
34. Tocanne, J.F., Tichadou, J.L., Lakhadar-Ghazal, F. 1983. Influence du pH et de la force ionique sur le degre d'ionisation de phospholipides acides dans des systemes modeles de membranes. *In:* Physical Chemistry of Transmembrane Ion Movements. G. Spach, editor. Elsevier, Amsterdam
35. Torrie, G.M., Valleau, J.P. 1982. Electrical double layers: 4. Limitations of the Gouy-Chapman theory. *J. Phys. Chem.* **86**:3251–3257
36. Wiersema, P.H., Loeb, A.L., Overbeek, J.T.G. 1966. Calculation of the electrophoretic mobility of a spherical colloid particle. *J. Colloid Interface Sci.* **22**:78–99

Received 14 March 1983

Appendix A

The Location of the Hydrodynamic Plane of Shear

The plane of shear was deduced to be 0.2 nm from the surface of a phospholipid vesicle formed in a 0.1 M monovalent salt solution by comparing the values of the surface and the zeta potentials and assuming that the dependence of the potential on distance is described correctly by double layer theory [2, 9]. When the monovalent salt concentration is reduced, the experimental values of the zeta potential do not change as much as predicted by the Gouy-Chapman-Stern theory if the plane of shear is assumed to remain 0.2 nm from the surface (Table). Similar results are obtained with all three cations: when the salt concentration is lowered from 0.1 to 0.01 M the predicted change in the zeta potential is about 50 mV whereas the observed change in the zeta potential is only about 40 mV. When the salt concentration is lowered from 0.01 to 0.001 M, the predicted change in the zeta potential is 58 mV whereas the observed change is again only about 40 mV. The reasons for these discrepancies are not clear. In order to describe the results we assume that the plane of shear shifts out from the surface as the monovalent salt concentration decreases. We stress, however, that other effects are likely to be important as well. McDonald and Bangham [19], Ohki [29] and Tocanne, Tichadou and Lakhdar-Ghazal [34] have investigated the dependence of the surface potential of a phospholipid monolayer on the monovalent salt concentration in the aqueous subphase. Their results agree qualitatively; the carefully controlled study of Tocanne et al. [34] demonstrates that the surface potential of a phosphatidylglycerol monolayer changes by only 53 rather than 58 mV when the [NaCl] changes by a factor of ten. These results suggest that the disagreement between the zeta potential results and the prediction of Gouy-Chapman-Stern theory is not solely due to a shift in the plane of shear from the surface.

Table. Zeta potentials of 5:1 PC/PS vesicles[a]

Salt concentration	Zeta potential, ζ (mV)		$\Delta\zeta$ (mV)	
	Experimental	Theoretical	Experimental	Theoretical
0.1 M NaCl	-28 ± 2	-28	41	49
0.01	-69 ± 2	-77	42	58
0.001	-111 ± 4	-135		
0.1 M CsCl	-31 ± 2	-35	44	50
0.01	-75 ± 1	-85	37	58
0.001	-112 ± 4	-143		
0.1 M TMACl	-40 ± 1	-36	42	51
0.01	-82 ± 1	-87	38	58
0.001	-120 ± 1	-145		

[a] The experimental data are taken from Figs. 2–10. The theoretical values were calculated from the Gouy-Chapman-Stern theory, Eqs. (1)–(4) of [9], by assuming that the association constants of Na, Cs and TMA with PS are 1.0, 0.1 and 0 M^{-1}, respectively, that the plane of shear is 0.2 nm from the surface of the membrane, and that both PS and PC occupy 0.7 nm^2 on the surface.

Appendix B

The Effect of Divalent Cations on the Surface Potential of PS Membranes

The addition of divalent cations to a solution containing PS membranes and a known free concentration of cobalt will affect the number of cobalt ions bound in inner sphere complexes with the phosphodiester group by two mechanisms. First, divalent cations will compete directly with cobalt ions for the phosphodiester binding site. Second, they will decrease the magnitude of the micropotential at the phosphodiester binding site, ψ_P. The second effect will decrease both the cobalt concentration and the sodium concentration near the binding site. We assume that monovalent and divalent cations compete for the same binding sites (i.e., the phosphodiester group and the carboxyl group). We also assume that only one cation can bind to a PS molecule. With these assumptions, the fraction of phosphodiester groups in PS molecules that are available to bind cobalt, β, is given by the following expression, a Langmuir isotherm [24]:

$$\beta=\frac{\alpha}{[1+K_1 C^{+}\exp(-F\psi_P/RT)+K_2 C^{++}\exp(-2F\psi_P/RT)]} \quad \text{(B1)}$$

where α is the fraction of PS molecules in the outer monolayer of the sonicated bilayer vesicles ($\alpha=0.67$; [20]), C^{+} and C^{++} are the monovalent and divalent cation concentrations in the bulk medium, K_1 and K_2 are the intrinsic monovalent and divalent cation association constants for PS [24] and ψ_P is the micropotential at the phosphodiester group. For simplicity, we assume that the micropotential at the phosphodiester group is the same as the micropotential at the carboxyl group.

The ratio of the ^{31}P NMR linewidth in the presence, $1/T_{2P}$, and absence, $1/T^{o}_{2P}$, of divalent cations is given by the expression

$$(1/T_{2P})/(1/T^{o}_{2P})=f/f^{o} \quad \text{(B2)}$$

where f and f^{o} are the fractions of phosphodiester groups involved in inner sphere complexes with cobalt in the presence and absence of divalent cations. If f is much less than one

$$f=\beta K_P\, Co^{++}(\infty)\exp(-2F\psi_P/RT) \quad \text{(B3)}$$

where $Co^{++}(\infty)$ is the cobalt concentration in the bulk medium and K_P is the intrinsic association constant for inner sphere complexes between cobalt and the phosphodiester group. The factor β complicates the calculation of $\Delta\psi_P$ using Eqs. (B1), (B2) and (B3). If we assume that β does not change on addition of divalent cations, Eqs. (B2) and (B3) can be combined to yield Eq. (4), which was used to provide one estimate of $\Delta\psi_P$ (*see* text). We examine the effect of this approximation on the calculation of $\Delta\psi_P$.

We assume that the intrinsic association constants for calcium, sodium, and dimethonium are 12 M^{-1}, 1 M^{-1} and 0 M^{-1}, respectively. The Gouy-Chapman-Stern theory then predicts a surface potential of -76 mV under the conditions of the NMR experiments, i.e., 0.1 M NaCl, 8 μM $CoCl_2$ [24]. As a first approximation, the values of ψ_P in the presence of 150 μM $CaCl_2$ (-66.8 mV) or 5 mM dimethonium (-71.5 mV) are calculated from the NMR data using Eq. (4). If we assume that calcium and sodium compete for the cobalt binding site, we calculate ψ_P from Eqs. (B1), (B2) and (B3) to be -65.1 and -69.5 mV. The two sets of points in Figs. 11*B* and 12*B* illustrate the values of $\Delta\psi_P$ calculated using the two different procedures.