# LARGE DIVALENT CATIONS AND ELECTROSTATIC POTENTIALS ADJACENT TO MEMBRANES

## Experimental Results with Hexamethonium

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ABSTRACT A simple extension of the Gouy-Chapman theory predicts that the ability of a divalent cation to screen charges at a membrane-solution interface decreases significantly if the distance between the charges on the cation is comparable with the Debye length. We tested this prediction by investigating the effect of hexamethonium on the electrostatic potential adjacent to negatively charged phospholipid bilayer membranes. The distance between the two charges of an extended hexamethonium molecule is ~1 nm, which is the Debye length in the 0.1 M monovalent salt solutions used in these experiments. Six different experimental approaches were utilized. We measured the electrophoretic mobility of multilamellar vesicles to determine the zeta potential, the line width of the $^{31}P$ nuclear magnetic resonance (NMR) signal from sonicated vesicles to calculate the change in potential at the phosphodiester moiety of the lipid, and the conductance of planar bilayer membranes exposed to either carriers (nonactin) or pore formers (gramicidin) to estimate the change in potential within the membrane. We also measured directly the effect of hexamethonium on the potential above a monolayer formed from negative lipids, and attempted to calculate the change in the surface potential of a bilayer membrane from capacitance measurements. With the exception of the capacitance calculations, each of the techniques gave comparable results: hexamethonium exerts a smaller effect on the potential than that predicted by the classic screening theory. The results are consistent with the predictions of the extended Gouy-Chapman theory and are relevant to the interpretation of physiological and pharmacological experiments that utilize hexamethonium and other large divalent cations.

## INTRODUCTION

Biological membranes contain charged lipids, sugars, and proteins, which produce an electrostatic potential and ionic double layer in the aqueous phase adjacent to the membrane. The simplest description of the diffuse double layer adjacent to a charged surface is that of Gouy (1) and Chapman (2), who combined the Poisson and Boltzmann equations. Stern (3) modified the theory by considering the

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adsorption of ions to the surface. In the Gouy-Chapman-Stern theory the ions in the diffuse double layer are assumed to be point charges. Monte Carlo calculations suggest that this assumption will not introduce serious errors if the diameters of the monovalent ions are 0.42 nm (4, 5); experimental results demonstrate that the classic theory describes adequately the effects of both the alkali metal and alkaline earth cations on the electrostatic properties of phospholipid bilayer membranes (6). The size of many physiologically and pharmacologically relevant ions, however, is too large to ignore. For example, the distance between the two positive charges of the ganglionic blocker hexamethonium is 1 nm, which is the Debye length in a decimolar salt solution. In the accompanying paper Carnie and McLaughlin (7) extend the Gouy-Chapman theory by taking into account the finite size of a divalent cation. They use the modified theory to predict the effect of a large divalent cation, such as hexamethonium, on the electrostatic potential adjacent to a phospholipid bilayer. In this paper we present the results of experimental tests of the extended theory.

## MATERIALS AND METHODS

### Electrophoretic Mobility Measurements

Brain phosphatidylserine (PS) and egg phosphatidylcholine (PC) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Salts were either ultrapure (Ventron Corp., Danvers, MA; Spex Industries, Inc., Metuchen, NJ) or analytical (Fisher Scientific Co., Fairlawn, NJ) grade. Decamethonium and hexamethonium were obtained from Sigma Chemical Co. (St. Louis, MO) and dimethonium was synthesized as described previously (8). The structures of these compounds are illustrated in Fig. 1. Water was purified with a Super-Q system (Millipore Corp., Bedford, MA), then double distilled in a quartz still. The concentrations of electrolytes were checked by measuring the conductivity of the solutions. All solutions were buffered to pH 7.5, 25°C, with [MOPS] = 0.01 [monovalent salt]. Multilamellar vesicles were formed by the method of Bangham et al. (9); electrophoretic mobilities were measured at 25°C with Rank Brothers Mark I microelectrophoresis machines (Bottisham, Cambridge, United Kingdom). (The Mark I machines proved superior in performance to the Rank Brothers Mark II machines for these experiments: settling of the vesicles on the bottom of the tube resulted in a more severe shift of the stationary layer in the Mark II machine because of the optical configuration.) Care was taken to focus at the stationary layer (10), and the current was monitored to ensure that electrode polarization did not occur. We circumvented the relaxation effect, which has been described theoretically (11) and experimentally (12), by observing only large (diameter >10 $\mu$m) vesicles when the salt concentrations was <0.1 M. The zeta potential, $\zeta$, was calculated from the electrophoretic mobility, $u$, using the Helmholtz-Smoluchowski equation

$$\zeta = u\eta/\epsilon_r\epsilon_o, \tag{1}$$

where $\epsilon_r$ is the dielectric constant of the aqueous phase, $\eta$ is the viscosity of the aqueous phase, and $\epsilon_o$ is the permittivity of free space. The zeta potential and surface potential results described below were compared with the predictions of the Gouy-Chapman-Stern theory (see Eqs. 3, 4, and 10 of reference 13).

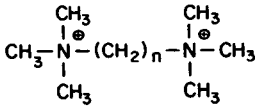

FIGURE 1 The chemical structures of dimethonium ($n$ = 2), hexamethonium ($n$ = 6), and decamethonium ($n$ = 10).

### $^{31}$P NMR Measurements

Low concentrations of paramagnetic divalent cations such as cobalt substantially increase the line width of the $^{31}$P NMR signal from sonicated phospholipid vesicles (14). This effect 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 immediately adjacent to the phosphate group. The free concentration is proportional to the Boltzmann factor, $\exp(-2F\psi_P/RT)$, where $\psi_P$ is the potential at the phosphodiester group. Low cobalt concentrations (<20 $\mu$M) affect neither $\psi_P$ (15) nor the zeta potential (13) of PS vesicles in 0.1 M NaCl; thus changes in the observed $^{31}$P NMR line width can be used to measure changes in the potential at the phosphodiester group upon the addition of cations such as hexamethonium. The ratio of the $^{31}$P NMR line width in the presence, $1/T_{2P}^{H}$, and absence, $1/T_{2P}^{O}$, of hexamethonium is given by the expression

$$\frac{1/T_{2P}^{H}}{1/T_{2P}^{O}} = \exp\{-2F\Delta\psi_P/RT\}, \tag{2}$$

where $\Delta\psi_P$ is the change in the potential at the phosphodiester group upon the addition of hexamethonium. The observed line widths were corrected by subtracting the natural line width and the small broadening introduced by trace amounts of paramagnetic impurities in the hexamethonium. The free concentrations of cobalt and hexamethonium were established by passing the sonicated PS sample through a Sephadex column. The calculation of $\Delta\psi_P$ from Eq. 2 assumes that the number of cobalt binding sites remains constant upon addition of hexamethonium. However, a substantial fraction of PS molecules are bound to sodium in 0.1 M NaCl (12), and the number of bound sodium ions is affected by changes in the surface potential. If sodium and cobalt compete for the same binding site, the addition of hexamethonium will alter the number of sites available to bind cobalt, and different equations must be used to calculate the effect of hexamethonium on the potential (8).

### Nonactin Conductance Measurements

Planar phospholipid bilayer membranes were formed from bovine brain PS (Supelco, Inc., Bellefonte, PA) monolayers as described previously (16). The hole in the Teflon partition of the chamber was pretreated with squalene, and the compartments of the chamber were connected to Ag/AgCl electrodes via 1 M KCl agar bridges. The solutions in both compartments were stirred during the addition of nonactin. When the conductance attained a steady state initial value, $G^0$, divalent cations were added to both compartments, the conductance, $G$, was measured again and the change in the electrostatic potential within the membrane, $\Delta\psi$, produced by the divalent cation was calculated from the equation

$$\Delta\psi = (-RT/F)\ln(G/G^0), \tag{3}$$

where $R$ is the gas constant, $T$ is the temperature, and $F$ is the Faraday constant (17, 18).

### Gramicidin Conductance Measurements

Planar lipid bilayers were formed from a dispersion of brain PS (Avanti Polar Lipids, Inc.) in hexadecane on a small hole in a polyethylene support between aqueous phases containing 0.1 M NaCl, 0.001 M MOPS, pH 7.5, and the desired concentration of hexamethonium bromide (Sigma Chemical Co.). All measurements were made at room temperature, 22°C. A potential of 100 mV was applied across the membrane via silver chloride/silver bromide electrodes and gramicidin A, which was purified from commercial gramicidin D (ICN Pharmaceuti-

cals, Inc., Cleveland, OH), was added to the aqueous phases in picomolar quantities until single-channel activity could be observed. The single-channel conductance was determined from the predominant peak, which was always well defined, in histograms of single-channel currents. Surface potentials, which alter the concentration of permeant ions near the mouth of the gramidicin channel, affect the conductance of these channels (19, 20). We estimated the change in the surface potential that influences the gramicidin channel, $\Delta\psi^{gram}$, from the ratio of single-channel conductances using the Boltzmann relation

$$\Delta\psi^{gram} = -(RT/F)\ln(G^{test}/G^{ref}), \quad (4)$$

where $G^{test}$ is the single-channel conductance in a solution containing hexamethonium, and $G^{ref}$ is the conductance in 0.1 M NaCl. This procedure probably underestimates the change in the surface potential because the conductance does not depend linearly on [NaCl] when the channel is in a neutral membrane. We also estimated the change in surface potential from

$$\Delta\psi^{gram} = -(RT/F)\ln(fG^{test}/G^{ref}), \quad (5)$$

where $f$ is a factor that corrects for the nonlinear dependence of the conductance on the concentration of sodium at the channel mouth. The correction factor, $f$, was calculated from the data of Neher et al. (21), which was obtained from gramicidin channels in bilayers formed from the neutral lipid monolein.[1]

## Monolayer Surface Potential Measurements

The surface potential of bovine brain PS (Supelco Inc.) monolayers was measured in a two compartment chamber (22). The subphase contained 0.1 M CsCl buffered to pH 7.2 with 1 mM MOPS. The area per molecule was 0.65 $nm^2$ for all monolayers. The change in the surface potential induced by the formation of the PS monolayer at the air/water interface was +320 ± 20 mV (air positive). We examined the effect of hexamethonium on the surface potential by replacing the subphase with one containing 0.1 M CsCl, 0.001 M MOPS, and the desired concentration of the divalent cation. All experiments were performed at room temperature (22 ± 2°C).

## Capacitance Measurements

Planar lipid bilayer membranes were formed as described by Alvarez and Latorre (16), who showed that the capacitance measured at an applied potential, $C(V)$, is described by

$$C(V) = C(0)\,[1 + \alpha(V + \Delta\psi)^2], \quad (6)$$

where $V$ is the applied potential, $C(0)$ is the minimal capacitance, $\alpha$ is a constant, and $\Delta\psi$ is the difference in the surface potential. A symmetric displacement in voltage, $\pm V_p$, around an arbitrary voltage value, $V_h$, produces a difference in $C(V)$, $\Delta C(V)$

$$\Delta C(V) = 6\alpha C(0) V_p (V_h - \Delta\psi). \quad (7)$$

We used the following pulse protocol to find $\Delta\psi$. The membrane potential was changed from zero to $V_h$ and held at that value for 250 μs; a pulse, $V_p$, duration 50 μs and magnitude either +50 mV or −50 mV was then added to $V_h$. 100 μs after the end of the pulse, the membrane potential was returned to zero. The currents were recorded during the pulses, $+V_p$ and $-V_p$. The currents were added and the time integral of the summed currents was divided by $V_p$ to obtain $\Delta C(V)$. The value of $\Delta\psi$ was determined from Eq. 7 by plotting $\Delta C(V)$ against $V_h$.

[1]To calculate the correction factor, $f$, from the data of Neher et al. (21), we estimated the concentration of sodium at the mouth of the channel from the Boltzmann relation using the experimentally determined values of the zeta potential in Fig. 4. This concentration is ~1 M; the exact value is irrelevant because of the shape of the curve.

# RESULTS

## Zeta Potential Measurements

The lower curve in Fig. 2 *A* illustrates the prediction of the Gouy-Chapman-Stern theory, assuming that the Cs-PS intrinsic association constant is 0.1 $M^{-1}$, the plane of shear is 0.2 nm from the surface (12), and the divalent cation-PS association constant is 0 $M^{-1}$. In other words, we assume that the divalent cation does not adsorb to PS. Even if it does not adsorb to the membrane, a point divalent cation should screen the charges and exert at least this effect on the zeta potential of the vesicles. The squares in Fig. 2 *A* illustrate the effect of hexamethonium on the zeta potential of PS vesicles formed in 0.1 M CsCl. Hexamethonium has a significantly smaller effect on the zeta potential than is predicted by the screening curve for a point divalent cation. This discrepancy is not due to adsorption of the divalent cation to the membrane because adsorption increases the effect of a cation on the zeta potential of a vesicle. For example, the diamonds in Fig. 2 *A* illustrate the effect of $Ca^{++}$ on the zeta potential of PS vesicles formed in 0.1 M CsCl. $Ca^{++}$ has a significantly larger effect on the zeta potential than that predicted by the screening curve. The upper curve in Fig. 2 *A* illustrates the prediction of the Gouy-Chapman-Stern theory as above, but assuming that the Ca-PS intrinsic association constant is 15 $M^{-1}$. The intrinsic 1:1 Ca-PS association constant is equal to the reciprocal of the concentration of $Ca^{++}$ required to cause charge reversal, $[Ca^{++}]_{rev}$ (13), which is

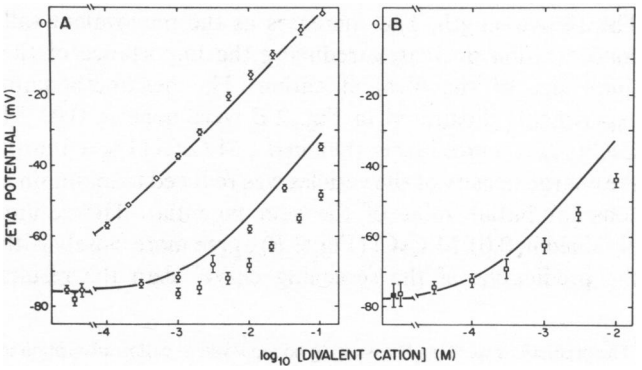


FIGURE 2 (*A*) The effect of hexamethonium (□), dimethonium (○), and calcium (◇) on the zeta potential of brain phosphatidylserine (PS) vesicles formed in 0.1 M CsCl. The *upper* curve illustrates the prediction of the Gouy-Chapman-Stern theory, assuming that the 1:1 Ca-PS intrinsic association constant is 15 $M^{-1}$. The *lower* curve illustrates the prediction of double-layer theory for a point divalent cation that does not adsorb to the membrane. Note that the hexamethonium data lie below this curve and that the dimethonium data are described well by the classic theory for a point charge. (*B*) The effect of hexamethonium (□) or 0.1 mM EDTA (■) on the zeta potential of 5:1 PC/PS vesicles formed in 0.01 M CsCl. The curve is the prediction of diffuse double-layer theory for a point divalent cation that does not adsorb to the membrane.

0.065 M in these experiments (Fig. 2 *A*). The theory describes adequately the effect of $Ca^{++}$ on the zeta potential of PS vesicles.

We hypothesize that the discrepany observed with hexamethonium is due to the finite size of the cation, i.e., the assumption that hexamethonium can be treated as a point charge is invalid. Carnie and McLaughlin (7) have modified the Gouy-Chapman theory to account for the finite size of divalent cations. Hexamethonium is modeled either as two point charges connected by an infinitely thin, rigid rod 1-nm long or as two noninteracting point charges connected by an infinitely thin, flexible string 1-nm long. The modified theory describes adequately the effect of hexamethonium on the zeta potential of PS vesicles.[2] For example, it predicts that addition of 0.01 M divalent cation will reduce the zeta potential of a PS vesicle to −69 mV (rod model) or −67 mV (string model; see Fig. 2 *A*, reference 7). In fact, addition of 0.01 M hexamethonium to PS vesicles reduces the zeta potential to −68 mV (Fig. 2 *A*). The excellent agreement between the experimental results and the theoretical predictions is either an accident or an example of what Stell (23) termed "the principle of unreasonable utility of asymptotic estimates."

Dimethonium is a short analogue of hexamethonium. The distance between the two charges is ~0.3 rather than 1 nm (Fig. 1). According to our hypothesis, the smaller dimethonium molecule should behave more like an ideal point divalent charge. The results of zeta potential measurements using dimethonium, illustrated by the circles in Fig. 2 *A*, confirm this prediction.

We tested our hypothesis that the discrepancy between the predictions of the Gouy-Chapman-Stern theory and the results obtained with hexamethonium is due to the finite size of the divalent cation in a second obvious way. The Debye length, $1/\kappa$, increases as the monovalent salt concentration decreases, reducing the importance of the finite size of the divalent cation. The hexamethonium experiments illustrated in Fig. 2 *B* were done in 0.01 M CsCl($1/\kappa \simeq 3$ nm) rather than in 0.1 M CsCl ($1/\kappa \simeq 1$ nm); the charge density of the vesicles was reduced to maintain a constant initial value of the zeta potential. The results obtained in 0.01 M CsCl (Fig. 2 *B*) agree more closely with the predictions of the screening curve[3] than the results obtained in 0.1 M CsCl (Fig. 2 *A*). The improvement in the fit is not striking, but is about that predicted by the modified Gouy-Chapman theory (see Fig. 2 *B*, reference 7). The effect of hexamethonium on the zeta potential of 5:1 PC/PS vesicles formed in 0.1 and 0.001 M CsCl is illustrated by the squares in Fig. 3 *A*, *B*. The curves illustrate the predictions of the screening theory for a point divalent cation (see footnote 3). When the monovalent salt concentration is high the Debye length is short ($1/\kappa \simeq 1$ nm), and we observe large deviations between the theoretical predictions and our results (Fig. 3 *A*). When the monovalent salt concentration is low the Debye length is long ($1/\kappa \simeq 10$ nm) and the deviations are proportionately smaller (Fig. 3 *B*). The results obtained in 0.001 M CsCl with the smaller dimethonium and the larger decamethonium molecules (Fig. 1), illustrated by the circles and triangles in Fig. 3 *B*, are also described qualitatively by the screening curve for a point divalent cation. We expected this result because all three divalent cations are small in comparison with the Debye length in a 0.001 M CsCl solution. The data presented in Figs. 2 and 3 are consistent with our hypothesis that the finite size of hexamethonium is responsible for this cation exerting a smaller screening effect than predicted for a point divalent cation.

We also examined the effect of hexamethonium on the zeta potential of phospholipid vesicles formed in NaCl. These results are more difficult to interpret theoretically because Na adsorbs more strongly than Cs to negative lipids such as PS; estimates of the intrinsic Na-PS association constant range from 0.6-1.0 $M^{-1}$ (12, 24, 25). The prediction of the Gouy-Chapman-Stern theory, assuming that the Na-PS association constant is 1 $M^{-1}$, the divalent cation-PS association constant is 0 $M^{-1}$, and the plane of shear is 0.2 nm from the surface, is illustrated by the curve in Fig. 4. The results obtained with hexamethonium (squares) lie below this curve; the results obtained with dimethonium (circles) are described well by the screening theory for a point divalent cation. We also measured the

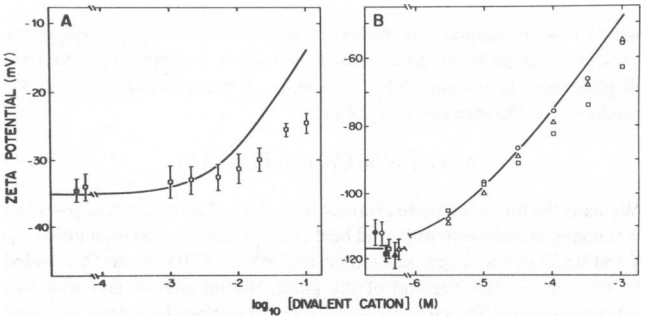


FIGURE 3 (*A*) The effect of hexamethonium (□) or 0.1 mM EDTA (■) on the zeta potential of 5:1 PC/PS vesicles formed in 0.1 M CsCl. (*B*) The effect of dimethonium (○), hexamethonium (□), decamethonium (△), and 1 μM EDTA (■, ●, ▲) on the zeta potential of 5:1 PC/PS vesicles formed in 0.001 M CsCl. The curves in both Fig. 3 *A* and *B* are the predictions of diffuse double-layer theory for a point divalent cation that does not adsorb to the membrane.

[2]The predictions were made assuming no monovalent cation adsorption to the membrane; the weak adsorption of Cs to PS has little effect on the predictions for divalent screening effects when the divalent salt concentration is >0.01 M. We used Cs for the experiments illustrated in Figs. 2 and 3 because it has the lowest binding constant to PS of the alkali metal cations (12).

[3]We assume that the Cs-PS intrinsic adsorption coefficient is 0.1 $M^{-1}$ and the divalent cation-PS adsorption coefficient is 0 $M^{-1}$. We also assume that the Gouy-Chapman-Stern theory describes exactly the dependence of the surface potential on the concentration of monovalent salt; this assumption is approximately correct (18, 35). To describe our initial data points, we assume that the plane of shear is 0.2, 0.4, or 1.0 nm from the surface in 0.1, 0.01, or 0.001 M monovalent salt solutions, as discussed in more detail in reference 13.

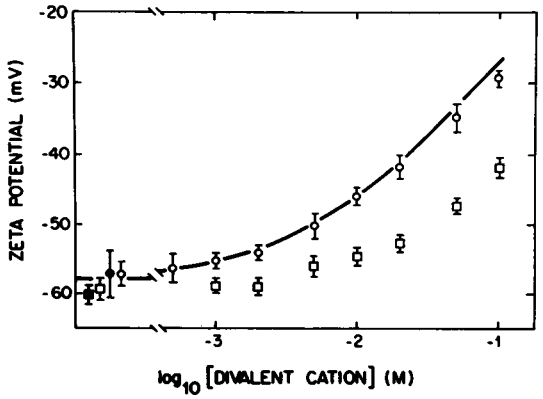


FIGURE 4 The effect of hexamethonium (□), dimethonium (○), and 0.1 mm EDTA (■, ●) on the zeta potential of PS vesicles formed in 0.1 M NaCl. The curve is the prediction of diffuse double-layer theory for a point divalent cation that does not adsorb to the membrane. Note that the dimethonium data are described well by the curve but that hexamethonium has a smaller effect than that predicted for a point divalent cation.

effect of dimethonium and hexamethonium on the electrophoretic mobility of 5:1 PC/PS vesicles formed in 0.1, 0.01, and 0.001 M NaCl solutions. The effect of dimethonium on the zeta potential was described well by the classical screening theory (8), whereas hexamethonium had a smaller effect than predicted by this theory (data not shown). These results are consistent with our hypothesis that the finite size of hexamethonium decreases its ability to screen the charges on phospholipid bilayer membranes.

The extended Gouy-Chapman theory (7) produces another testable prediction: the salt of a large divalent cation and a small anion should produce a negative zeta potential on vesicles with no net charge. The concentration of the large divalent cation is lower at the surface of the membrane than in the bulk aqueous phase because the surface reduces the configuration available to the cation, a phenomenon termed "entropic repulsion" (7). The excess anions adjacent to the membrane should produce a negative space charge and a negative zeta potential. Table I presents the results obtained when hexamethonium bromide was added to a solution containing vesicles formed from PC, a zwitterionic phospholipid. The zeta potential of PC vesicles in 0.1 M NaCl is zero (column 1); addition of 0.1 M hexamethonium bromide produces a zeta potential of −6 mV. About −2 mV of this potential is probably due to adsorption of the $Br^-$ ion (column 4, Table I); the remaining −4 mV is presumably due to the entropic repulsion of hexamethonium from the surface of the membrane. The Gouy-Chapman theory, which assumes that the divalent cations are point charges, predicts that the potential should be zero. The extended theory, which models the divalent cation as a 1-nm rod, predicts that the zeta potential should be −2 mV, a number that agrees reasonably well with the experimental result.

TABLE I
EFFECT OF HEXAMETHONIUM AND DECAMETHONIUM ON THE ZETA POTENTIAL* OF PC VESICLES FORMED IN 0.1 M NaCl

| | 0 M | 0.01 M | 0.1 M | 0.2 M |
|---|---|---|---|---|
| Hexamethonium bromide | +0.6 ± 0.7 | −0.7 ± 0.4 | −6.4 ± 0.8 | — |
| Sodium bromide | +0.6 ± 0.7 | — | — | −2.4 ± 0.8 |
| Decamethonium bromide | +0.6 ± 0.7 | −0.1 ± 0.5 | −0.0 ± 0.8 | — |

The concentration of the bromide salt in the solution is indicated in the first line.
*Millivolts ± SD; $n \geq 20$.

Not all the zeta potential results we obtained are consistent with our hypothesis. The extended theory (7) predicts that decamethonium should produce a more negative potential than hexamethonium on a vesicle that bears no net charge. We obtained the opposite result: addition of 0.1 M decamethonium bromide to a solution containing PC vesicles produced no zeta potential (Table I). The extended theory also predicts that the larger decamethonium molecule should exert a smaller effect than hexamethonium on the zeta potential of negatively charged vesicles. Again, we obtained the opposite result.[4] The observation that decamethonium is more flexible than hexamethonium (26) suggests an explanation for this discrepancy: decamethonium may, in some of its conformations, adsorb hydrophobically to the bilayer membranes.

## Nuclear Magnetic Resonance Measurements

Electrophoretic mobility measurements allow us to determine the electrostatic potential at the hydrodynamic plane of shear; $^{31}P$ NMR measurements allow us to determine changes in the electrostatic potential at the phosphate moiety of the lipid. Low concentrations of paramagnetic cations broaden the observed $^{31}P$ NMR signal from PS membranes, and this effect can be used to measure changes in the potential at the phosphodiester group induced by the addition of diamagnetic ions (14). The squares in Fig. 5 *A* illustrate the effect of hexamethonium on the $^{31}P$ NMR line width of sonicated PS vesicles prepared in 0.1 M NaCl. The aqueous solutions also contained a free cobalt concentration of 8 μM. The squares in Fig. 5 *B* illustrate the change in the electrostatic potential at the phosphodiester group, $\Delta\psi_P$, produced by hexamethonium. $\Delta\psi_P$ was calculated in two different ways. The

[4]We observed that decamethonium always had a larger effect than hexamethonium on the zeta potential. We repeated the experiments illustrated in Figs. 2 *A*, *B* and 3 *A* with decamethonium. Similarly, when we repeated the experiments illustrated in Figs. 2 and 3 with NaCl rather than CsCl solutions, we observed that hexamethonium always had a smaller effect on the zeta potential than predicted by the classic screening theory (e.g., Fig. 4), and that decamethonium had a larger effect on the zeta potential than hexamethonium. All the results obtained with decamethonium fell between the hexamethonium results and the predictions of the screening theory.

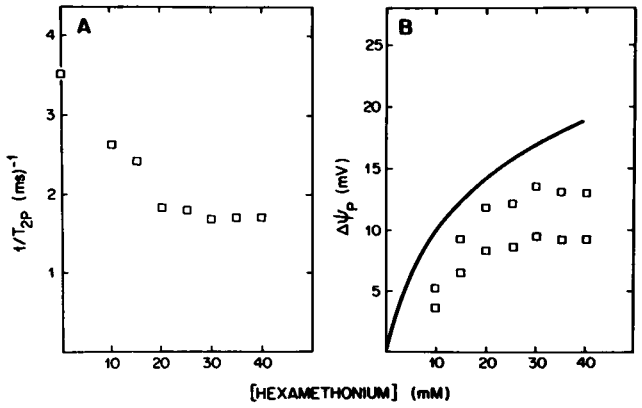


FIGURE 5 (*A*) The effect of hexamethonium on the $^{31}P$ NMR line width, $1/T_{2P}$, of PS molecules in the outer monolayer of sonicated vesicles in 0.1 M NaCl, 8 $\mu$M free $Co^{++}$, and 1 mM MES buffered to pH 5.0, 25°C. (*B*) The change in the potential at the phosphodiester group, $\Delta\psi_P$, produced by hexamethonium. The *lower* set of squares (□) was determined from the corresponding points in Fig. 5 *A* using Eq. 2. The *upper* set of squares (□) was determined by assuming that sodium and cobalt compete for the same binding site. The curve is the prediction of diffuse double-layer theory for a point divalent cation that does not adsorb to the membrane.

lower value for each pair of squares in Fig. 5 *B* was calculated from the corresponding datum in Fig. 5 *A* using Eq. 2. The upper value for each pair of squares in Fig. 5 *B* was calculated by assuming that sodium and cobalt ions compete for for the same binding site and that the intrinsic Na-PS association constant is 1 $M^{-1}$ (8). The curve in Fig. 5 *B* illustrates the change in the surface potential predicted by the Gouy-Chapman-Stern theory, assuming the Na-PS intrinsic association constant is 1 $M^{-1}$ and the divalent cation-PS association constant is 0 $M^{-1}$. Hexamethonium has a smaller effect on the potential at the phosphodiester group than is predicted by this screening curve for a point divalent cation. This result is consistent with our hypothesis that the finite size of this cation decreases its ability to screen negative charges. The NMR results agree well with the zeta potential results. For example, addition of 10 mM hexamethonium produces a change of 4–5 mV in both the zeta potential of a PS multilamellar vesicle (Fig. 4) and the potential at the phosphodiester group of a PS unilamellar vesicle (Fig. 5 *B*).

## Nonactin-induced Conductance Measurements

The experiments presented thus far illustrate how divalent cations change the electrostatic potential both in the aqueous phase adjacent to the membrane and at the phosphodiester group of the lipid molecule. We investigated the effect of divalent cations on the electrostatic potential within the hydrocarbon interior of a planar PS membrane formed in 0.1 M CsCl by making conductance measurements: the change in the electrostatic potential within the membrane, $\Delta\psi$, was calculated from the nonactin-induced conductance using Eq. 3, the Boltzmann relation. The squares in Fig. 6 illustrate the effect of hexamethonium on this potential (left-hand ordinate). The lower curve in Fig. 6 illustrates the surface potential, $\psi_0$ (right-hand ordinate), or the change in surface potential, $\Delta\psi$ (left-hand ordinate), predicted by the Gouy-Chapman-Stern theory assuming that the Cs-PS intrinsic association constant is 0.1 $M^{-1}$ and the divalent cation-PS association constant is 0 $M^{-1}$. Hexamethonium has a smaller effect on $\psi_0$ than predicted by this screening curve for a point divalent cation, a result that is consistent with our conclusions from zeta potential and NMR experiments. The diamonds in Fig. 6 illustrate the effect of $Ca^{++}$ on $\Delta\psi$; the upper curve illustrates the change in surface potential predicted by the Gouy-Chapman-Stern theory, assuming the Cs-PS and the Ca-PS intrinsic association constants are 0.1 and 15 $M^{-1}$, respectively. The same association constants were used to describe the zeta potential results presented in Fig. 2. The theory provides an adequate description of the effects of $Ca^{++}$ on the surface potential of planar lipid bilayers.

We obtained results similar to those illustrated in Fig. 6 when PS membranes were formed in 0.1 M KCl (Fig. 7). Zeta potential measurements indicate that $K^+$ adsorbs slightly more strongly to PS than does $Cs^+$. The K-PS intrinsic association constant was calculated to be 0.15 $M^{-1}$ (12). The effect of $Ca^{++}$ on $\Delta\psi$ (diamonds, Fig. 7) is described well by the Gouy-Chapman-Stern theory, assuming that the K-PS intrinsic association constant is 0.15 $M^{-1}$ and the intrinsic Ca-PS association constant is 15 $M^{-1}$ (upper curve, Fig. 7). The lower curve in Fig. 7 illustrates the predictions of the theory if the divalent cation-PS association constant is assumed to be 0 $M^{-1}$;

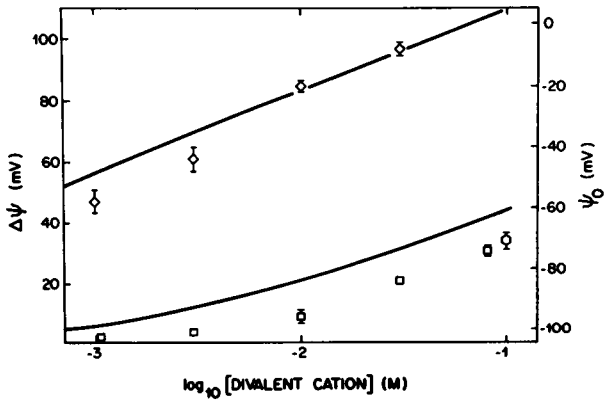


FIGURE 6 The change in the electrostatic potential within a planar PS bilayer membrane, $\Delta\psi$ (left-hand ordinate), produced by calcium (◇) and hexamethonium (□). $\Delta\psi$ was calculated from the conductance using Eq. 3. The solutions contained 0.1 M CsCl, 0.33 $\mu$M nonactin, and 0.001 M MOPS buffered to pH 7.0 at 22°C. The hexagon illustrates the effect of 0.1 M hexamethonium on the surface potential of a PS monolayer formed over a 0.1 M CsCl solution.

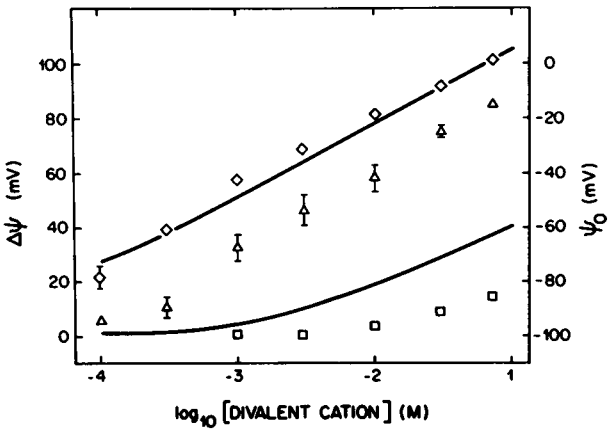


FIGURE 7 The change in the electrostatic potential within a planar PS bilayer membrane, $\Delta\psi$ (left-hand ordinate), produced by calcium (◇), decamethonium (△), and hexamethonium (□). The solutions contained 0.1 M KCl, 0.33 μM nonactin, and 0.001 M MOPS buffered to pH 7.0 at 22°C.

hexamethonium (squares, Fig. 7) has a smaller effect on $\Delta\psi$ than predicted by this screening curve for a point divalent cation. Decamethonium (triangles, Fig. 7) has a much larger effect on $\Delta\psi$ than predicted by the screening curve. The effect of decamethonium on the potential in the center of a membrane (Fig. 7) is much larger than its effect on the potential at the hydrodynamic plane of shear (see footnote 4).

## Monolayer Measurements

The hexagon in Fig. 6 illustrates the change in the surface potential above a PS monolayer produced by adding 0.1 M hexamethonium bromide to the subphase, which contained 0.1 M CsCl ($n = 3$, ± SD). The result is consistent with the $\Delta\psi$ measured in the nonactin-conductance experiments.

## Gramicidin Conductance Measurements

Table II lists the effects of hexamethonium bromide on the main peak conductance of a single gramicidin channel.

TABLE II
EFFECT OF HEXAMETHONIUM BROMIDE ON THE CONDUCTANCE OF A SINGLE GRAMICIDIN CHANNEL IN A PS BILAYER FORMED IN A 0.1 M NaCl SOLUTION

| [Hexamethonium] | Conductance | $\Delta\psi^{gram}$ | |
|---|---|---|---|
| | | (Eq. 4) | (Eq. 5) |
| *mM* | *pS* | *mV* | |
| 0 | 15.6 | 0.0 | 0.0 |
| 1 | 14.9 | 1.0 | 1.5 |
| 3 | 14.4 | 2.1 | 3.6 |
| 10 | 13.1 | 4.4 | 8.0 |
| 30 | 12.5 | 6.0 | 11.9 |
| 100 | 10.9 | 9.1 | 20.3 |

The addition of hexamethonium to the aqueous phase reduces the single-channel conductance (column 2, Table II). The change in the surface potential calculated from Eq. 4, the Boltzmann relation, is listed in column three of Table II. The change in the surface potential calculated from Eq. 5, which corrects for the nonlinear dependence of conductance on [NaCl], is listed in column four of Table II. We compared these two estimates of the change in surface potential sensed by gramicidin (Table II, columns 3 and 4) with the curve in Fig. 5 *B*, which illustrates the predictions of the Gouy-Chapman-Stern theory. We conclude that hexamethonium has a smaller effect on the gramicidin conductance than a hypothetical point divalent cation that only exerts a screening effect. Although the gramicidin results are qualitative because of the large correction factor, they do agree well with the zeta potential (Fig. 4) and NMR (Fig. 5) measurements.

## Capacitance Measurements

Membrane capacitance, which is proportional to the reciprocal of membrane thickness, should be minimal when the transmembrane potential, the electrostatic potential between the two membrane solution interfaces, is zero. The transmembrane potential will be zero when the applied potential is equal to the difference between the surface potentials of the two constituent monolayers, $\Delta\psi$. We estimated the value of $\Delta\psi$ produced by the addition of a divalent cation to one side of a membrane by measuring the applied potential that minimized the capacitance. When we used this method to study the effect of calcium on the surface potential of the PS planar bilayer membranes formed in 0.1 M NaCl, we obtained results (data not shown) that agreed well with nonactin conductance measurements on planar bilayers, with zeta potential and NMR measurements on vesicles, and with surface potential measurements on monolayers. However, when we used the capacitance technique to study the effects of hexamethonium and decamethonium on the surface potential of PS bilayers, we obtained anomalous results. A concentration of 0.1 M hexamethonium, for example, produced an apparent change of 90 ± 8 mV in the surface potential of a PS membrane in 0.1 M NaCl, a value much larger than those estimated by the other techniques. The discrepancy was even larger for decamethonium: 0.001 M decamethonium, for example, produced an apparent change of 85 ± 10 mV in the surface potential of a PS membrane in 0.1 M NaCl. The reason for these anomalous results is not clear.

## DISCUSSION

Our main conclusions are that hexamethonium has a smaller effect on the electrostatic potential adjacent to phospholipid membranes than predicted by the classical screening theory, that the deviations are due to the finite

size of the cation, and that the results can be accounted for by an extended Gouy-Chapman theory (7). We have presented results from five different experimental systems that are consistent with these conclusions. The advantages and disadvantages of each technique we utilized are discussed below in the context of our interpretation of the measurements.

The zeta potential results we obtained with dimethonium, a short analogue of hexamethonium, provide the best evidence that the deviations from the predictions of the classical theory observed with hexamethonium are due to the finite size of this molecule. While the effect of hexamethonium on the electrophoretic mobility of phospholipid vesicles is smaller than predicted by the classical screening theory, the effect of dimethonium is described well by the classical theory (Figs. 2, 4), which assumes that the cation can be treated as a point charge. The results obtained with hexamethonium are consistent with the predictions for a point divalent cation only when the monovalent salt concentration is very low, and the Debye length very long (Fig. 3 *B*). However, all the electrophoretic mobility measurements with hexamethonium are qualitatively consistent with the predictions of the extended Gouy-Chapman theory (7). Electrophoretic mobility measurements allow us to ascertain the potential, rather than just changes in the potential, at the hydrodynamic plane of shear adjacent to a phospholipid vesicle. The disadvantage of this technique is that the position of the plane of shear is not well defined. We have argued elsewhere (12) and present additional evidence below, that the plane of shear is ~0.2 nm from the surface of a phospholipid vesicle in a decimolar monovalent salt solution. However, the available evidence is insufficient to allow us to make a strong argument about its position in solutions of lower ionic strength.

We measured the effect of hexamethonium on the potential at the phosphodiester group of phospholipids with $^{31}P$ NMR. The results agree well with the zeta potential measurements. Hexamethonium has a smaller effect on the potential than predicted for a point divalent cation (Fig. 5). The main advantage of this technique is that it provides information about the change in the electrostatic potential at a precisely defined location, the phosphodiester group. One disadvantage of this technique is that the calculation of $\Delta\psi_P$ requires assumptions about the competition between the monovalent ions and divalent ions for the phosphate binding sites (8).

The advantages and disadvantages of the nonactin-induced conductance technique have been discussed (17, 18). The nonactin conductance measurements allow us to calculate directly only the change in the electrostatic potential within the hydrocarbon interior of the membrane. We can, however, combine these measurements with charge reversal zeta potential measurements to estimate the surface potential. For example, 0.03 M $Ca^{++}$ changes the potential within a planar PS bilayer formed in 0.1 M CsCl by about +100 mV (Fig. 6) and reduces the zeta potential of a PS vesicle formed in 0.1 M CsCl almost to zero (Fig. 2 *A*). We conclude that the surface potential[5] of a PS membrane formed in 0.1 M CsCl must be about −100 mV, as indicated on the right-hand ordinate of Fig. 6.

This estimate of the surface potential can be used to test our assertion that the hydrodynamic plane of shear is 0.2 nm from the surface in decimolar salt solutions. The zeta potential of a PS vesicle formed in 0.1 M CsCl is −75 mV (Fig. 2 *A*); the surface potential is −105 mV (Fig. 6). If we assume that the potential profile adjacent to a phospholipid bilayer can be described by the Gouy-Chapman theory, an assumption that is qualitatively correct (27), we calculate (Eq. 4, reference 12) that the plane of shear is 0.20 nm from the surface. Our observation that the Gouy-Chapman-Stern theory predicts the effect of $Ca^{++}$ on both the zeta potential (Fig. 2) and the surface potential (Figs. 6, 7) of bilayer membranes supports our assertion that the model, although highly oversimplified, provides a surprisingly accurate description of the effect of small divalent cations on the electrostatic potential.

A comparison of conductance and zeta potential measurements also provides support for our suggestion that decamethonium adsorbs to bilayer membranes. Decamethonium has a much larger effect on the potential within a PS membrane (e.g., Fig. 7) than on the zeta potential under comparable conditions (see footnote 4). Our interpretation of this result is that decamethonium adsorbs to the membrane and changes the dipole potential. (Decamethonium will also affect the diffuse double layer potential by both changing the charge density and exerting a screening effect.) The available evidence suggests that the electrophoretic mobility of vesicles does not respond to changes in the dipole potential at the membrane-solution interface, whereas both the nonactin conductance of planar membranes and the surface potential of monolayers do respond to changes in the dipole potential (18, 28). The same argument leads us to postulate that calcium does not change significantly the dipole potential of PS membranes. The effects of calcium on the electrophoretic mobility of PS vesicles (Fig. 2; reference 13), the nonactin conductance of PS bilayers (Fig. 6, 7), and the surface potential of PS monolayers (29) are described well by the Gouy-Chapman-Stern theory, assuming a 1:1 Ca-PS intrinsic association constant of 10–15 $M^{-1}$.

The monolayer results provide information comparable with the nonactin-induced conductance measurements (Fig. 6). The advantage of this technique is that we can measure directly changes in the potential. Two main

[5]The surface potential is defined to be the potential in the aqueous phase at the membrane-solution interface and does not include any contributions from molecular dipoles at this interface. The potential in the bulk aqueous phase is defined to be zero. The dipole potential at the interface of a phospholipid bilayer is estimated to be several hundred millivolts, interior positive (28, 36).

disadvantages are that the lipids are in a different molecular configuration, a monolayer rather than a bilayer, and that a large dipole potential of unknown molecular origin arises when a phospholipid monolayer is spread (28).

The estimates of the surface potential we obtained from the gramicidin single-channel measurements are consistent with the results obtained using the other techniques discussed above. There are two main disadvantages to using this technique to estimate the change in surface potential. First, we do not know what fraction of the surface potential affects the gramicidin conductance. Second, the conductance does not depend linearly on [NaCl] when the gramicidin channel is in a neutral membrane and large correction factors are necessary to calculate the change in potential.

We do not understand why the capacitance measurements allow us to deduce correctly the effect of $Ca^{++}$ but not the effects of hexamethonium and decamethonium on the surface potentials of bilayer membranes. The capacitance does not depend strongly on the applied potential when measurements are made on solvent-free membranes. Thus, any factor that affects the capacitance will introduce an artifact into these measurements.

Physiologists and pharmacologists are generally not concerned about the effect of high concentrations of large divalent cations on the surface potential of membranes: hexamethonium, for example, is pharmacologically active at concentrations that have no effect on the surface potential (30). Nevertheless our conclusion that hexamethonium exerts a smaller effect on the electrostatic potential of bilayer membranes than predicted by classical screening theory, and our demonstration that this effect can be predicted by an extended Gouy-Chapman theory that considers the finite size of the ion (7), do have biological implications. There is now good evidence that negative electrostatic potentials exist at the acetylcholine receptors in muscle and at the sodium channels in nerve; these negative potentials will increase the local concentration of a cationic drug, and any factor that influences these potentials will also influence the apparent affinity of the receptor for the drug. Several groups have commented on the significance of this observation (31, 32, 33), but they have all assumed that large divalent cations, such as saxitoxin and curare, are point charges in their quantitative treatments. The results presented above demonstrate that this is not a valid assumption. As discussed in more detail in Carnie and McLaughlin (7), the finite size of a large divalent cation has a significant effect on its concentration adjacent to a negatively charged membrane and on the apparent affinity of the receptor for the cation. This conclusion should also be of interest to investigators who are following Miller's elegant use of large divalent cations as structural probes of ion channels (34).

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## REFERENCES

1. Gouy, M. 1910. Sur la constitution de la charge électrique a la surface đun électrolyte. *J. Physiol. (Paris).* 9:457–468.
2. Chapman, D. L. 1913. A contribution to the theory of electrocapillarity. *Phil. Mag.* 25:475–481.
3. Stern, O. 1924. Zur Theorie der elektrolytischen Doppelschicht. *Z. Elektrochem.* 30:508–516.
4. Torrie, G. M., and J. P. Valleau. 1980. Electrical double layers. I. Monte Carlo study of a uniformly charged surface. *J. Chem. Phys.* 73:5807–5816.
5. Valleau, J. P., and G. M. Torrie. 1982. The electrical double layer. III. Modified Gouy-Chapman theory with unequal size ions. *J. Chem. Phys.* 76:4623–4630.
6. McLaughlin, S. 1982. Divalent cations, electrostatic potentials, bilayer membranes. *In* Membranes and Transport. A. Martinosi, editor. Plenum Press, New York. 1:51–55.
7. Carnie, S., and S. McLaughlin. 1983. Large divalent cations and electrostatic potentials adjacent to membranes. A theoretical calculation. *Biophys. J.* 44:325–332.
8. McLaughlin, A., W.-K. Eng, G. Vaio, T. Wilson, and S. McLaughlin. 1983. Dimethonium, a divalent cation that exerts only a screening effect on the electrostatic potential adjacent to negatively charged phospholipid bilayer membranes. *J. Membr. Biol.* In press.
9. Bangham, A. D., M. W. Hill, and N. G. A. Miller. 1974. Preparation and use of liposomes as models of biological membranes. *Methods Membr. Biol.* 1:1–68.
10. Henry, D. C. 1938. A source of error in micro-cataphoretic measurements with a cylindrical bore cell. *J. Chem. Soc.* (*Lond.* ). 997–999.
11. Overbeek, J. Th. G., and P. H. Wiersema. 1967. The interpretation of electrophoretic mobilities. *In* Electrophoresis: Theory, Methods, and Applications. M. Bier, editor. Academic Press, Inc., New York. 1–52.
12. Eisenberg, M., T. Gresalfi, T. Riccio, and S. McLaughlin. 1979. Adsorption of monovalent cations to bilayer membranes containing negative phospholipids. *Biochemistry.* 18:5213–5223.
13. McLaughlin, S., N. Mulrine, T. Gresalfi, G. Vaio, and A. McLaughlin. 1981. Adsorption of divalent cations to bilayer membranes containing phosphatidylserine. *J. Gen. Physiol.* 77:445–473.
14. Lau, A., A. McLaughlin, and S. McLaughlin. 1981. The adsorption of divalent cations to phosphatidylglycerol bilayer membranes. *Biochim. Biophys. Acta.* 645:279–292.
15. McLaughlin, A. C. 1982. Phosphorous-31 and carbon-13 nuclear magnetic resonance studies of divalent cation binding to phosphatidylserine membranes: use of cobalt as a paramagnetic probe. *Biochemistry.* 21:4879–4885.
16. Alvarez, O., and R. Latorre. 1978. Voltage-dependent capacitance in lipid bilayers made from monolayers. *Biophys. J.* 21:1–17.
17. Szabo, G., G. Eisenman, S. G. A. McLaughlin, and S. Krasne. 1972. Ionic probes of membrane structure. *Ann. NY Acad. Sci.* 195:273–290.
18. McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interfaces. *Curr. Top. Membr. Transp.* 9:71–144.
19. Szabo, G., and D. McBride. 1978. Influence of double-layer and dipolar surface potentials on ionic conductance of gramicidin channels. *Biophys. J.* 21:25*a*. (Abstr.)

20. Apell, H.-J., E. Bamberg, and P. Lauger. 1979. Effects of surface charge on the conductance of the gramicidin channel. *Biochim. Biophys. Acta.* 55:369–378.
21. Neher, E., J. Sandbloom, and G. Eisenman. 1978. Ionic selectivity, saturation and block in gramicidin A channels. *J. Membr. Biol.* 40:97–116.
22. Reyes, J., and R. Latorre. 1979. Effect of the anesthetics benzyl alcohol and chloroform on bilayers made from monolayers. *Biophys. J.* 28:259–280.
23. Stell, G. 1977. Fluids with long-range forces: toward a simple analytic theory. *In* Statistical Mechanics. Part A: Equilibrium Techniques. B. J. Berne, editor. Plenum Press, New York. 47–84.
24. Nir, S., C. Newton, and D. Papahadjopoulos. 1978. Binding of cations to phosphatidylserine vesicles. *Bioelectrochem. Bioenerg.* 5:116–133.
25. Kurland, R., C. Newton, S. Nir, and D. Papahadjopoulos. 1979. Specificity of $Na^+$ binding to phosphatidylserine vesicles from a $^{23}Na$ NMR relaxation study. *Biochim. Biophys. Acta.* 551:137–147.
26. Dufourcq, J., B. Clin, and B. Lemanceau. 1972. NMR study of ganglion-blocking and curare-like dimethoniums. Conformation in aqueous solutions. *FEBS (Fed. Eur. Biochem. Soc.).* 22:205–209.
27. Loosley-Millman, M. E., R. P. Rand, and V. A. Parsegian. 1982. Effects of monovalent ion binding and screening on measured electrostatic forces between charged phospholipid bilayers. *Biophys. J.* 40:221–232.
28. Hladky, S. B. 1974. The energy barriers to ion transport by nonactin across thin lipid membranes. *Biochim. Biophys. Acta.* 352:71–85.
29. Ohki, S., and R. Kurland. 1981. Surface potential of phosphatidylserine monolayer. II. Divalent and monovalent binding. *Biochim. Biophys. Acta.* 645:170–176.
30. Wiley, R. A., B. A. Faraj, and A. Jantz. 1972. Influence of stereochemistry lipophilicity on biological activity of some ganglionic blocking agents. *J. Med. Chem.* 15:374–378.
31. Henderson, R., J. M. Ritchie, and G. R. Strichartz. 1974. Evidence that tetrodotoxin and saxitoxin act at a metal cation binding site in the sodium channels of nerve membrane. *Proc. Natl. Acad. Sci. USA.* 71:3936–3940.
32. Lewis, C. A., and C. F. Stevens. 1979. Mechanism of ion permeation through channels in a postsynaptic membrane. *In* Membrane Transport Processes. C. F. Stevens and R. W. Tsien, editors. Raven Press, New York. 3:89–103.
33. Van der Kloot, W. G., and I. Cohen. 1979. Membrane surface potential changes may alter drug interactions: an example, acetylcholine and curare. *Science (Wash., DC).* 203:1351–1353.
34. Miller, C. 1982. *Bis*-quaternary ammonium blockers as structural probes of the sarcoplasmic reticulum $K^+$ channel. *J. Gen. Physiol.* 79:869–891.
35. Tocanne, J. F., J. L. Tichadou, and F. Lakhdar-Ghazal. 1983. Influence du pH et de la force ionique sur le degre d'ionisation de phospholipides acides dans des systemes models de membranes. *In* Physical Chemistry of Transmembrane Ion Movements. G. Spach, editor. Elsevier, Amsterdam.
36. Andersen, O. S., and M. Fuchs. 1975. Potential energy barriers to ion transport within lipid bilayers. *Biophys. J.* 15:795–830.