Divalent Ions and the Surface Potential of Charged Phospholipid Membranes

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ABSTRACT   Phospholipid bilayer membranes were bathed in a decimolar solution of monovalent ions, and the conductance produced by neutral carriers of these monovalent cations and anions was used to assess the electric potential at the surface of the membrane. When the bilayers were formed from a neutral lipid, phosphatidylethanolamine, the addition of alkaline earth cations produced no detectable surface potential, indicating that little or no binding occurs to the polar head group with these ions. When the bilayers were formed from a negatively charged lipid, phosphatidylserine, the addition of Sr and Ba decreased the magnitude of the surface potential as predicted by the theory of the diffuse double layer. In particular, the potential decreased 27 mv for a 10-fold increase in concentration in the millimolar-decimolar range. A 10-fold increase in the Ca or Mg concentration also produced a 27 mv decrease in potential in this region, which was again due to screening, but it was necessary to invoke some specific binding to account for the observation that these cations were effective at a lower concentration than Ba or Sr. It is suggested that the ability of the alkaline earth cations to shift the conductance-voltage curves of a nerve along the voltage axis by 20–26 mv for a 10-fold increase in concentration may be due to essentially a screening rather than a binding phenomenon.

INTRODUCTION

The stimulus for this study was the well-known observation (Frankenhaeuser and Hodgkin, 1957) that an increase in the concentration of divalent ions in the solution bathing a nerve or muscle produces a shift in the conductance-voltage curves along the voltage axis in the positive direction; that is, ions like Ca^{++} have a stabilizing influence on the nerve. Included in Frankenhaeuser and Hodgkin's (1957) initial report of the phenomenon was the suggestion by A. F. Huxley that Ca^{++} might bind to the outer surface of the nerve and thereby produce a local hyperpolarization or additional electric field within

the membrane capable of influencing the field-dependent Na and K channels. Another simple mechanism whereby divalent cations could produce the shifts observed on nerve and muscle is illustrated in Fig. 1. If the negative charge density, σ, on the membrane is high, there will be a negative potential at the surface of the membrane relative to the bulk aqueous phase (Fig. 1 a). The theory of the diffuse double layer predicts that the addition of even a low concentration of divalent ions to one side of the membrane will reduce the negative surface potential on that side by a screening process (Fig. 1 b). This will
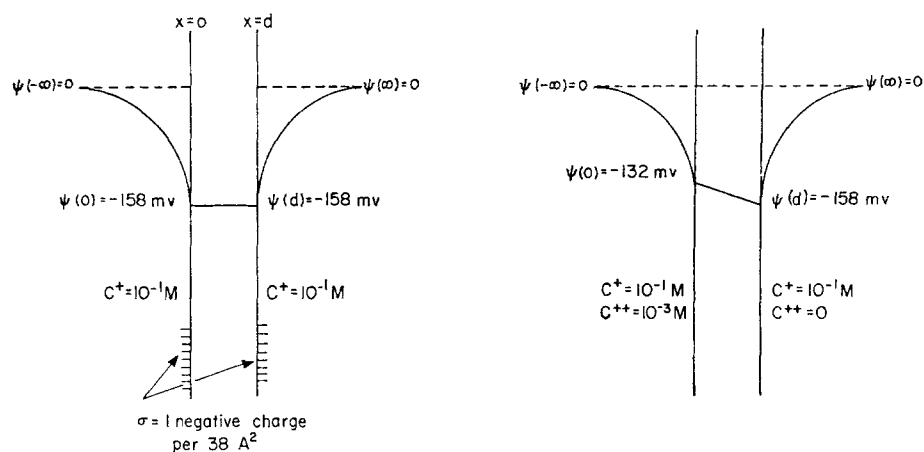

Figure 1. (a) Schematic diagram of the electric potential profile near a charged membrane. The potential at the surface of the membrane is that predicted by the theory of the diffuse double layer or Gouy equation for the indicated values of the charge density and indifferent electrolyte concentration. (b) The effect of divalent cations on the surface potential in the absence of any specific adsorption. The decrease in potential is that predicted by the theory of the diffuse double layer if the divalent cation is impermeant. Note that the divalent cations produce a substantial ($\cong RT/F \cong$ 25 mv) reduction in the surface potential by screening the charges even though the "ionic strength" of the solution remains essentially constant.

produce a potential difference across the membrane, which could affect the voltage-dependent sodium and potassium channels.

With the exception of Gilbert and Ehrenstein (1969), who did consider the possibility that Ca++ could screen as well as bind to negative sites on a squid axon, the simple possibility that divalent ions may affect the surface potential, and hence shift the conductance-voltage curves of nerve by a screening mechanism, has been generally ignored, possibly because divalent ions affect the nerve at such low concentrations that the "ionic strength" is maintained as essentially a constant by the monovalent ions. The concept of ionic strength, however, should be applied with caution to a biological membrane, for it does not correctly predict the relative screening effects of monovalent and divalent

counterions at a highly charged surface, as will be discussed below. The Gouy-Chapman or diffuse double layer theory does describe adequately the potential at a simple charged surface like a mercury dropping electrode (e.g. Delahay, 1965) but there are several theoretical objections to applying it to a biological membrane (e.g. Cole, 1969). The main objective of this study, therefore, was to test experimentally whether the theory could account for the effects of divalent ions on the surface potential of a model membrane.

A previous study (McLaughlin et al., 1970) illustrated that the conductance produced by neutral carriers of monovalent cations and anions could be used to assess the potential at the surface of bilayer membranes. The theory of the diffuse double layer accounted qualitatively for the surface potential of a bilayer formed from a negatively charged bacterial lipid as the divalent ions Ca and Mg were added to the aqueous phases containing 10^{-3} M KCl. We report here the effects of the alkaline earth cations on the surface potential of bilayer membranes formed from the major charged lipid of nerve, phosphatidylserine (Camejo et al., 1969), and one of the major amphoteric lipids, phosphatidylethanolamine, when such bilayers are bathed in a "physiological" solution of monovalent ions (10^{-1} M KCl, pH 7.2). The double layer theory is shown to predict correctly both the relative screening abilities of the monovalent and divalent ions and the slope of the surface potential vs. concentration curves. This study also illustrates how binding can be distinguished from screening and examples are given of divalent cations which affect the surface potential of phosphatidylserine bilayers by screening (Sr), both screening and binding (Ca), and binding alone (UO_2). The results reported here complement a recent study (Muller and Finkelstein, manuscript in preparation) on artificial membranes involving the voltage-dependent ion translocator, monazomycin. When this molecule is introduced into a negatively charged bilayer and divalent cations added to one side of the membrane, the conductance-voltage curves are shifted along the voltage axis by essentially a screening mechanism.

MATERIALS AND METHODS

The apparatus and procedure used to obtain conductance measurements were similar to those described by Szabo et al. (1969). Bilayers were formed on a hole about 1 mm² in area in a partition which separated two aqueous phases. The solutions were always of identical composition and contained initially 10^{-1} M KCl buffered to pH 7.2 with 2×10^{-3} M tris(hydroxymethyl)amino methane. Preliminary experiments on phosphatidylserine and phosphatidylglycerol bilayers formed in the presence and absence of the buffer indicated that this concentration of Tris had no effect on the conductance produced by the neutral carriers. The data reported here were obtained from single experiments, but duplicate experiments were performed in all cases and this data could always be fitted by the empirical curve drawn through the first set of points.

Membranes were formed from mixtures of n-decane and the following lipids (a) 7-dehydrocholesterol (Sigma Chemical Co., St. Louis, Mo.), which is neutral; (b) a cyclopropane-rich phosphatidylethanolamine, extracted from $E.$ $coli$ (Supelco, Inc., Bellefonte, Pa.) which is neutral but amphoteric; (c) a cyclopropane-rich phosphatidylglycerol (Supelco, Inc., Bellefonte, Pa.) which has one negative charge; and (d) a phosphatidylserine of animal origin, the fatty acid residues of which contain double bonds and which also has one negative charge (identical results were obtained on a sample from Nutritional Biochemicals Corporation, Cleveland, Ohio and Calbiochem, Los Angeles, Calif.). The negative lipids were shaken in ether with an aqueous solution of 10^{-2} M H_2SO_4 to remove any traces of polyvalent cations.

RESULTS

1. *The Surface Potential in the Absence of Divalent Ions*

In the presence of a neutral carrier of cations such as the antibiotic nonactin, the conductance, G^+, of a charged bilayer membrane has been shown (Neumcke, 1970; McLaughlin et al., 1970) to be equal to the conductance of a neutral membrane times the exponent of the potential at the surface of the membrane, $\psi(0)$:

$$(G_0^+)^{\text{charged}} = (G_0^+)^{\text{neutral}} \exp \frac{-F\psi(0)}{RT}. \tag{1}$$

The subscript zero in equation 1 indicates that the conductance is to be measured in the limit of zero applied voltage and $RT/F = 25.3$ mv at 22°C. For a negatively charged permeant species such as the polyiodide complex:

$$(G_0^-)^{\text{charged}} = (G_0^-)^{\text{neutral}} \exp \frac{+F\psi(0)}{RT}. \tag{2}$$

These equations are valid only if the products of the mobility and partition coefficient of the complex are the same for the charged and neutral membranes, an assumption which is verified experimentally below.

The rationale behind using conductance measurements to deduce the surface potential of bilayer membranes is apparent from equation 1, and such measurements are illustrated in Fig. 2 a. The bilayers were formed from decane solutions of a neutral lipid, phosphatidylethanolamine (PE), and of two negatively charged lipids, phosphatidylserine (PS) and phosphatidylglycerol (PG). As aliquots of nonactin were added to the aqueous phases bathing the membrane the conductance increased linearly with the nonactin concentration, in agreement with the previous observations of Szabo et al. (1969) and McLaughlin et al. (1970) made at lower salt concentrations. The linear increase is expected if the permeant species is the known 1:1 complex (Kilbourn et al., 1967; Eisenman et al., 1969) formed between a nonactin molecule and a potassium ion.

To estimate the potential at the surface of the negatively charged membranes we require the ratio $(G_0^+)^{charged}/(G_0^+)^{neutral}$, or, as the data in Fig. 2 are presented on a log scale, the difference of log G_0^+ at a given nonactin concentration. It is apparent from Fig. 2 a that the nonactin-induced conductance of the negatively charged membranes is 2.7 log units greater than that of the neutral bilayer and that the difference is independent of the nonactin concentration.

As a control, we then examined the conductance of bilayers formed from
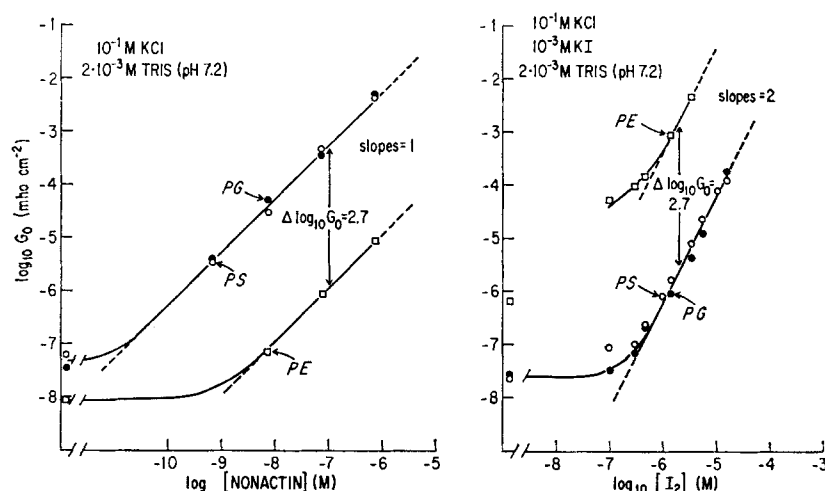

Figure 2. (a) The effect of the antibiotic nonactin on the conductance of a neutral (PE) and two negatively charged (PS and PG) phospholipid bilayer membranes. The permeant species is the positively charged nonactin-K^+ complex. (b) The effect of the neutral iodine molecule on the conductance of bilayers formed from the same lipids. The permeant species is the negatively charged I_5^- complex. The change of 2.7 log units in the conductance implies from equations 1 and 2 that the potential at the surface of the negatively charged membranes is -158 mv.

these same lipids produced by a negative permeant species, the I_5^- complex formed between two iodine molecules and an iodide ion (Fig. 2 b). As in Fig. 2 a, the membranes were bathed in 10^{-1} m KCl, but with the addition of 10^{-3} m KI to provide an anion capable of complexing with the neutral iodine. As iodine was added to the aqueous phase the conductance increased with the square of the iodine concentration, consistent with the notion that the permeant species is the I_5^- complex formed between two iodine molecules and an iodide ion (Finkelstein and Cass, 1968; McLaughlin et al., 1970). Fig. 2 b is symmetrical with respect to Fig. 2 a; with a positive permeant species the conductance of the negative membranes is enhanced 2.7 log units, with a negative permeant species it is depressed 2.7 log units relative to the neutral membrane.

From equations 1 and 2 and the conductance data of Fig. 2 we deduce that the potential at the surface of the negatively charged membranes is $(-2.7)58.5 = -158$ mv. This is a reasonable potential in terms of diffuse double layer theory, as a consideration of the Gouy expression indicates (see equation 2 a; sinh $[F\psi(0)/2RT] = 136\ \sigma/\sqrt{C}$). Inserting the value of $\psi(0) = -158$ mv, $C = 10^{-1}$ M, one obtains the value $\sigma = 1$ negative charge/38 Å².[1]

2. Variation of the Surface Potential on the Addition of Divalent Ions

(A) PHOSPHATIDYLSERINE BILAYERS   The main purpose of this study was to test experimentally whether divalent ions affect the surface potential of a charged bilayer membrane in accordance with the predictions of the diffuse double layer theory. This confrontation of theory and experiment is illustrated in Fig. 3, where the bilayers have been formed from the negatively charged lipid, phosphatidylserine. In the upper portion of the figure the left-hand ordinate designates the measured change in G_0^+, whereas the right-hand ordinate plots the surface potential inferred from equation 1 as a function of the divalent ion concentration. The bathing solutions contain 10^{-1} M KCl, and $\psi(0)$ in the absence of divalent ions is therefore -158 mv (see Fig. 2). The solid line is the pure screening curve deduced from the Graham equation (Appendix 1.) for a negative surface charge density of $\sigma = 1/38$ Å², or, equivalently, an initial surface potential of -158 mv. This is the initial value deduced from Fig. 2, and it should be stressed that there are no other adjustable parameters in this curve.

It is apparent in Fig. 3 that the Sr^{++} and Ba^{++} data agree very well with this curve. When the divalent ion concentration, C^{++}, is greater than 10^{-3} M the magnitude of the surface potential decreases about 27 mv for a 10-fold increase in divalent ion concentration and the full theoretical curve may be approximated by the simple Gouy expression for divalent ions alone, sinh $F\psi(0)/RT \cong -1/2\exp -F\psi(0)/RT = 136\ \sigma/\sqrt{C^{++}}$.

The dashed line is the theoretical curve which includes the possibility that the divalent cations can bind to, or form ion pairs with, the negative sites on the membrane as well as screening them by the formation of a diffuse double layer (see Appendix, 1.). A best fit to the Ca^{++} and Mg^{++} data was obtained with an association constant $K = 0.1$ liter/mole (visually better than $K =$

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[1] This value agrees qualitatively with data obtained from force-area measurements on monolayers (Van Deenen et al., 1962) and X-ray scattering measurements on bilayers (Levine and Wilkins, 1971), both of which indicate that the area per phospholipid molecule in a bilayer should lie in the range between 40 and 60 Å². The charge density at the surface of the bilayers required by double layer theory to produce the potential of -158 mv is thus slightly higher than what might be expected from direct measurements on bilayers and monolayers, but in view of the assumptions inherent in the derivation of the Gouy equation it is extremely gratifying that a reasonable value is obtained.
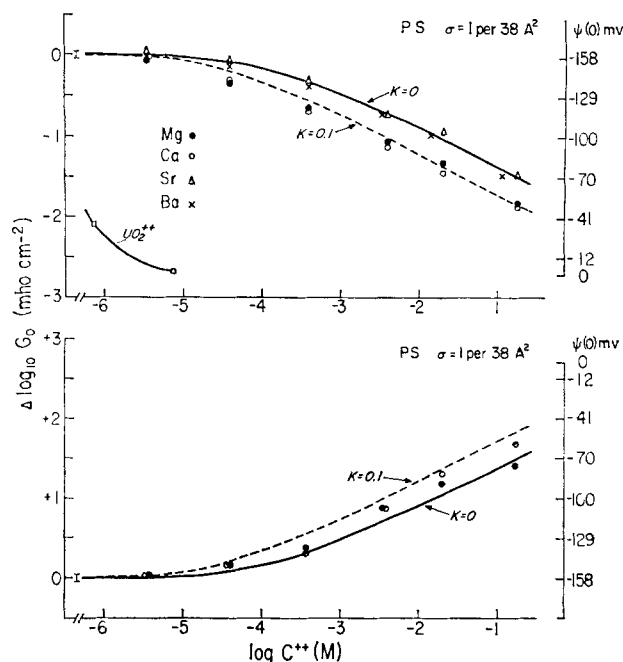
Figure 3.   (a) The effect of divalent cations on the surface potential of a bilayer membrane formed from the negatively charged lipid, phosphatidylserine. The left-hand ordinate plots the measured change in the conductance due to a positive permeant species (the nonactin-K^+ complex) as divalent ions are added symmetrically to the aqueous phases. The right-hand ordinate designates the surface potential deduced from such measurements via equation 1. The aqueous phase contains initially 10^{-1} M KCl, 6×10^{-7} M nonactin, and 2×10^{-3} M Tris buffered to pH 7.2. The vertical bar is twice the standard error of the mean of the absolute value of the initial conductances. The solid line designated by $K = 0$ is the theoretical curve for the surface potential predicted from double layer theory if no binding of the ions occurs, whereas the dashed line designated by $K = 0.1$ is the theoretical curve for the surface potential which includes the possibility that divalent cations may bind to negative sites on the membrane with an association constant $K = 0.1$ liter/mole as well as screening the charges. (b) The effect of calcium and magnesium on the surface potential of a PS bilayer membrane, as deduced from the conductance produced by a negative permeant species, the polyiodide complex. The aqueous phase contains 10^{-1} M KCl, 10^{-3} M KI, 2×10^{-3} M Tris buffered to pH 7.2, and 2×10^{-6} M I_2. The curves and ordinates are labeled as above. Note the conductance increases as divalent ions are added to the aqueous phases.

0.075 or $K = 0.25$). This low association constant implies that half of the sites on the bilayers are bound when the concentration at the interface is 10 M.

One point should be stressed with respect to the theoretical curves in Fig. 3. When $10^{-3} < C^{++} < 10^{-1}$ M, the decrease in $\psi(0)$ with an increase in C^{++} is due in both cases to a screening process and not to any further binding of divalent ions. This is because the number of bound ions is a function of a single

variable, the free concentration of ions at the interface, which is determined by the Boltzmann relation, the product of the bulk concentration, and the exponent of twice the surface potential in units of RT/F. As the bulk concentration, C^{++}, is increased, the screening effect reduces the potential by the amount required to maintain the product a constant. The free, and thus also the bound, concentration of ions is therefore independent of the bulk concentration in this range, as is shown in Appendix, *3*.

The association constants of the alkaline earth cations with PS are all extremely low (<0.1 liter/mole) and, as discussed above, the decrease they produce in the magnitude of the surface potential may be mainly attributed to a screening process. This, however, is not the case with all divalent ions as the data for the uranyl ion, UO_2^{++}, in Fig. 3 illustrate. UO_2^{++} affects G_0^+ at extremely low concentrations, and when the concentration has increased to a value between 10^{-6} and 10^{-5} M, G_0^+ has decreased about 2.7 log units, which implies that the surface potential has decreased from -160 to 0 mv. This decrease in $\psi(0)$ must be attributed to a binding of UO_2^{++} to the membrane because the screening effect of a divalent ion is still negligible at a concentration of 10^{-5} M (see solid curve in Fig. 3).

The lower portion of Fig. 3 is a control experiment which illustrates the effect of divalent ions on the conductance of PS bilayers produced by a negative permeant species, the I_5^- complex. The experiments are difficult to perform because the neutral carrier, the iodine molecule, is continually being lost from the aqueous solution, presumably because it is being taken up by the double bonds in the hydrocarbon tails of the excess lipid present in the torus surrounding the membrane. Although the scatter in the G_0^- data (lower half Fig. 3) is much greater than in the G_0^+ data (upper half Fig. 3), the Ca^{++} and Mg^{++} curves are symmetrical to a first approximation. G_0^- increased whereas G_0^+ decreased with an increase in C^{++}, confirming the assumption that the change in conductance was produced by a change in the surface potential.

Further confirmation of this assumption comes from a direct measurement of the change in the surface potential of a PS monolayer upon addition of alkaline earth cations to the aqueous subphase. When the subphase contained monovalent ions at a concentration of 0.145 M, Papahadjopoulos (1968) observed that Ca^{++} at a concentration of 10^{-4} M produced about a 20 mv change in the surface potential, and that a 10-fold increase in the concentration of Ca^{++} in the range between 10^{-4} and 10^{-2} M produced about a 27 mv change in the surface potential. These observations are in quantitative agreement with the results of Fig. 4, which were deduced from conductance measurements made on a PS bilayer.

(B) PHOSPHATIDYL GLYCEROL BILAYERS   Fig. 4 presents the results of experiments analogous to those illustrated in Fig. 3, but with bilayers formed from the negatively charged lipid phosphatidylglycerol (PG). The results

obtained with PG resemble those obtained with PS membranes in that the alkaline earth cations produce a decrease in G_0^+ and an increase in G_0^- which may be accounted for qualitatively by a pure screening expression (Fig. 4, solid lines).

Considered in more detail, the data in Fig. 4 differ in two features from those in Fig. 3. On PG bilayers only Ca^{++} has a significantly greater effect
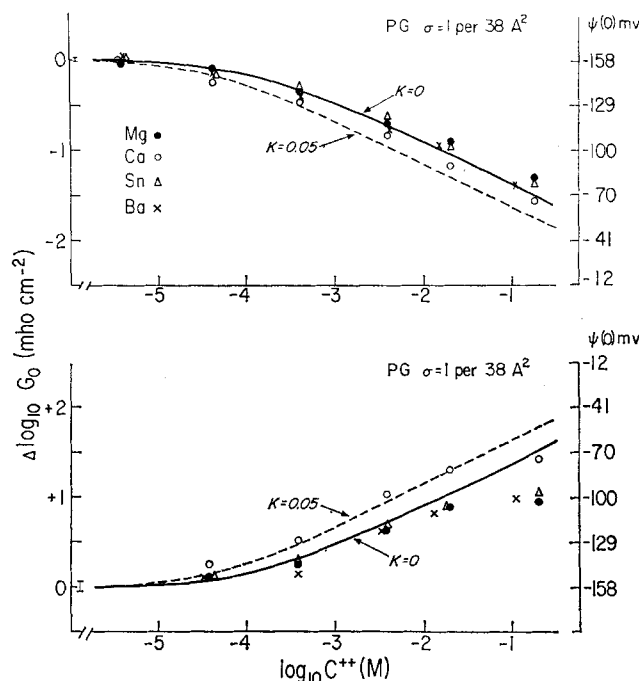

Figure 4. The effect of the alkaline earth cations on the surface potential of a bilayer membrane formed from the negatively charged lipid phosphatidylglycerol. The initial composition of the aqueous solutions was identical to that described in the legend of Fig. 3; the ordinate and short horizontal bars also have the same significance. The solid line designated $K = 0$ is, as in Fig. 3, the theoretical expression for the potential from diffuse double layer theory, whereas the curve designated as $K = 0.05$ includes the possibility that divalent cations bind to negative sites on the membrane with an association constant $K = 0.05$ liter/mole as well as screening the charges.

than predicted by a screening theory (solid curve) and only for this ion is it necessary to postulate that ion pair formation occurs with negative sites on the bilayer. Furthermore, the deviations from the theoretical curves with PG (Fig. 4) are greater than with PS (Fig. 3). The main effect of divalent ions is still, however, to decrease the magnitude of the negative surface potential and there is no necessity to postulate a strong binding of the alkaline earth cations to explain this effect.

(c) 7-DEHYDROCHOLESTEROL BILAYERS   At concentrations between 10^{-6}

and 10^{-1} M, neither Ca^{++} nor Mg^{++} affects the conductances produced on a 7-dehydrocholesterol bilayer by the permeant nonactin-potassium and poly-iodide complexes. This was expected because 7-dehydrocholesterol is a neutral molecule which has neither charges to screen nor sites with which the ions are expected to associate and the experiment may be regarded as a control which demonstrates that the alkaline earth cations do not themselves affect the permeant species. Uranyl up to a concentration of 10^{-4} M and thorium up to a
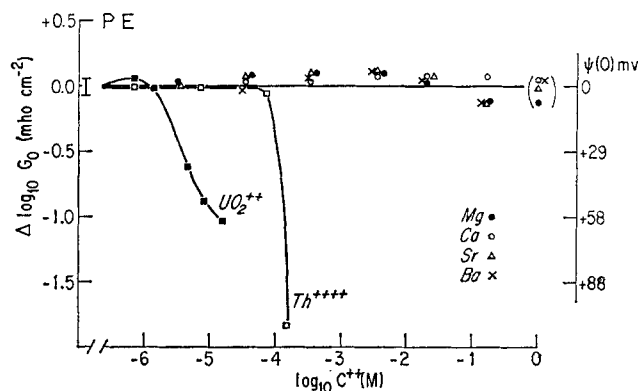

FIGURE 5. The effect of polyvalent cations on the conductance of a phospholipid bilayer formed from the neutral lipid phosphatidylethanolamine. The solution contains initially 10^{-1} M KCl, 6×10^{-7} M nonactin, and 2×10^{-3} M Tris at pH 7.2. The vertical bar designates twice the standard error of the mean of the absolute value of the initial conductances. As in the previous two figures, the left-hand ordinate plots the measured change in the conductance whereas the right-hand ordinate plots the surface potential deduced from equation 1. Note that all the alkaline earth cations have little effect on the surface potential up to a concentration of 10^{-1} M. The data obtained at a concentration of 1 M are in parentheses and should be regarded with caution because the divalent ions themselves produce a conductance comparable to that induced by the nonactin-K^+ complex at these high concentrations. Note that the uranyl and thorium ions produce a positive surface potential.

concentration of 10^{-3} M also produce no changes in the conductance induced by nonactin, an experiment which may be regarded as a control for the effects of UO_2^{++} observed on the negative PS membrane (Fig. 3) and for the effects of both of the ions on a membrane formed from the neutral but amphoteric lipid, phosphatidylethanolamine (Fig. 5.).

(D) PHOSPHATIDYLETHANOLAMINE BILAYERS   Fig. 5 illustrates the effects of various impermeant polyvalent cations on the nonactin-induced conductance of a bilayer membrane formed from the neutral but amphoretic lipid phosphatidylethanolamine (PE). It is apparent from Fig. 5 that increasing the concentration of the alkaline earth cations to 10^{-1} M produces no significant change in G_0^+, hence no change in the inferred surface potential, which should

be 0 mv for this neutral lipid. This implies that the association constants of the alkaline earth cations with the phosphate moiety in the polar head group of PE are less than 1 liter/mole, a conclusion consistent with the association constants deduced for these ions with the phospholipids PS and PG. There should, of course, be no screening or double layer effects on this neutral lipid.

The divalent ion UO_2^{++}, which was strongly bound to bilayers formed from the negative lipid PS (Fig. 3), also binds to the amphoteric lipid PE and produces a positive surface potential at a concentration between 10^{-6} and 10^{-5} M, in agreement with the previous observations of Bangham et al. (1967). The order of magnitude of the association constants required to explain the effects of UO_2^{++} are about 10^5 liter/mole for PE and about 10^6 liter/mole for PS. The highly polarizable thorium ion, which exists in a variety of charged forms in an aqueous solution, also binds strongly to the PE bilayer (Fig. 5) and the association constant required to explain its effect is about 10^4 liter/mole.

Thus, there are polyvalent cations such as uranyl and thorium which will bind to even a neutral lipid like PE, but what we wish to stress from Fig. 5 and the preceding Figs. 3 and 4 is that the alkaline earth cations have extremely low association constants with the phospholipids PS, PG, and PE. Binding is only observed with membranes formed from the negative lipids PS and PG because the aqueous concentration of divalent ions at the surface of these bilayers is several orders of magnitude higher than the concentration in the bulk aqueous solution. The results of a recent study utilizing the fluorescent dye ANS, however, have been used to argue that inorganic cations are strongly bound to a neutral lipid (Gomperts et al., 1970), but an alternative explanation is suggested by the data presented in the next section.

3. The Surface Potential Produced by 1-Anilino-8-naphthalenesulfonate (ANS)

The upper portion of Fig. 6 illustrates that the negatively charged ANS molecules adsorb to a neutral membrane at low concentrations and produce a large negative surface potential. ANS also has the property of fluorescing in media of low dielectric constant, and the fluorescence in a solution of phospholipid vesicles is presumably due to the dye molecules adsorbed at the lipid-water interface because the apolar residue of ANS penetrates for a short distance into the hydrocarbon core of the bilayer (Lesslauer et al., 1971). Vanderkooi and Martonosi (1969) observed that the fluorescence from such a solution was enhanced on addition of inorganic ions and demonstrated that this was due to an increased number of adsorbed ANS molecules. Divalent cations were found to be about an order of magnitude more effective than monovalent cations (10^{-3} vs. 10^{-2} M) in enhancing the ANS fluorescence in a solution containing vesicles formed from the neutral phospholipids PE or PC, and trivalent cations were about an order of magnitude more effective than divalent cations (10^{-4} vs. 10^{-3} M). They stated that the increase of fluorescence "cannot be attributed

to general ionic strength effects since the ionic strength of KCl and MgCl₂ which produce similar enhancements of fluorescence differs by at least one order of magnitude" and Gomperts et al. (1970) contended that the effect was due to the association of inorganic cations with the phospholipids. As discussed
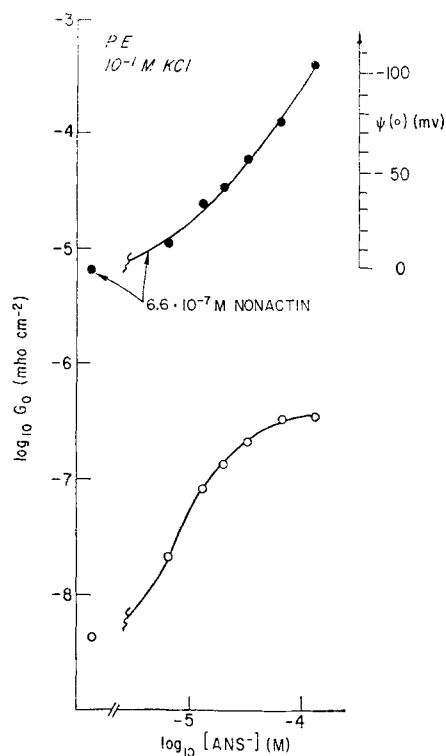

FIGURE 6. The surface potential produced by the fluorescent dye ANS on a bilayer membrane formed from the neutral lipid phosphatidylethanolamine. The lower curve (open circles) designates the conductance produced by ANS in the absence of nonactin. The upper curve (filled circles) illustrates the effect of ANS on the conductance produced by the positively charged nonactin-K⁺ complex. Note that the conductance due to ANS is always two orders of magnitude lower then the conductance produced by the neutral carrier, but that this latter conductance is nevertheless markedly enhanced by ANS. This increase is expected if ANS adsorbs to the surface of the membrane and produces a negative surface potential and the increase in G_0^+ can be used to calculate the surface potential (see equation 1), which is plotted on the right-hand ordinate.

in Appendix, 2., however, the concept of "ionic strength" should not be applied to a charged membrane when ions of different valence are considered unless the potential at the surface of the membrane is less than 25 mv. The observations can be explained in terms of double layer theory if sufficient ANS molecules are adsorbed onto the vesicle membranes to transform them from a neutral to a negatively charged bilayer. We suggest that the adsorption of ANS

produces a negative surface potential, which repels the negatively charged ANS molecules in the solution from the surface of the membrane and that the addition of ions merely "screens" the surface charges and allows more ANS to be adsorbed while the surface potential and surface concentration in the aqueous phase remain approximately constant. By invoking the three Gouy equations (2 a):

$$\sinh \frac{zF\psi(0)}{2RT} = \frac{136\sigma}{\sqrt{C}}$$

$$z = 1, 2, 3 \quad \text{for} \quad C^+, C^{++}, \text{ and } C^{+++}$$

and assuming that the same number of ANS molecules are adsorbed to a neutral PE membrane at concentrations of mono-, di-, and trivalent ions $C^+ = 10^{-2}$ M, $C^{++} = 10^{-3}$ M, $C^{+++} = 10^{-4}$ M, the concentrations required to produce similar enhancements of the fluorescence, we find that a surface potential of about $\psi(0) = -60$ mv will explain *both* the relative effects of monovalent vs. divalent *and* divalent vs. trivalent ions. Fig. 6 provides experimental evidence which indicates that the "screening" rather than the "binding" hypothesis is the correct explanation. At the concentration of ANS which the above investigators used in their experiments, 5 or 6 $\times$ 10^{-5} M, ANS does adsorb to the surface and produce a potential slightly in excess of -60 mv, in quantitative agreement with the prediction of a screening hypothesis.

DISCUSSION

1. *The Applicability of Double Layer Theory to Phospholipid Bilayers and Biological Membranes*

The results presented here demonstrate that the simplest form of diffuse double layer theory, due originally to Gouy and to Chapman (e.g. Delahay, 1965), is capable of accounting for the screening effects of both monovalent and divalent ions on the surface potential of charged phospholipid bilayer membranes, as inferred from conductance measurements. A decade change in the divalent ion concentration in the range $10^{-3} < C^{++} < 10^{-1}$ M produces a 27 mv change in the surface potential, $d\psi(0)/d\log C^{++} = 27$ mv, as predicted by the Graham equation (1 a) (Figs. 3 and 4). Furthermore, the theory predicts correctly the relative screening effects of monovalent and divalent ions (see equation 6 a). For a membrane with one electronic charge per 40–60 Å^2, the screening effects of divalent ions at a concentration $C^{++} \cong 2 \times 10^{-4}$ M should be equal to those of monovalent ions at a concentration $C^+ = 10^{-1}$ M (Figs. 3 and 4), and the screening effects of divalent ions at a concentration $C^{++} \cong 10^{-7}$ M dominate those of monovalent ions at a concentration $C^+ = 10^{-3}$ M (McLaughlin et al., 1970).

This agreement with the simple theory is perhaps surprising, in view of the many assumptions inherent in the derivation of the Graham equation. As these assumptions have been discussed in detail by Haydon (1964), Davies and Rideal (1963), and Barlow (1970), we will merely list the three which should introduce the most serious errors in concentrated salt solutions. The charge on the membrane is assumed to be uniformly distributed over the surface (Cole, 1969), the dielectric constant in the aqueous phase is assumed to be a constant and equal to its bulk value, and the ions are assumed to be point charges. As discussed by the above investigators, the simple double layer theory should be applicable to most surfaces, as indeed we find it is for the bilayer membranes, not so much because the errors introduced by these assumptions are negligible but because they should tend to cancel one another out. This fortuitous cancellation of secondary effects on a phospholipid bilayer membrane implies that one can now apply the theory of the diffuse double layer to biological membranes with more confidence than in the past (e.g. Chandler et al., 1965).

2. The Association Constants of the Alkaline Earth Cations with the Phospholipids PS, PG, and PE

The results reported here imply that the association constants of the alkaline earth cations with the phospholipids PS, PG, and PE (Figs. 3–5) are all quite low (<1 liter/mole).[2] The values of the association constants deduced for the negatively charged lipids should be regarded as an order of magnitude estimate because of the assumptions inherent in the theory of the diffuse double layer, some of which were noted in the previous section. The lack of effect of the alkaline earth cations on the neutral lipid PE (Fig. 5), however, does support the claim that the association constants with the phosphate group are less than unity.

The data we obtained with charged lipids are consistent with those of Abramson et al. (1964 a) who measured the release of hydrogen from a dispersion of PS as calcium was added to the aqueous phase. Abramson et al. (1968) also observed a release of H^+ upon addition of Ca^{++} to a phos-

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[2] Up to a concentration of 1 M, these cations have no effect on the surface potential of a bilayer formed from the neutral lipid PE. Even with an association constant of only 0.1 liter/mole, however, Ca^{++} does significantly reduce the charge density of PS bilayers at a bulk concentration between 10^{-5} and 10^{-3} M (Fig. 3, compare the solid line, which is the pure screening curve, and the dashed line which includes ion association), but this is because the free concentration at the surface of the bilayer, as predicted by the Boltzmann equation, $C(0) = C(-\infty) \exp - 2F\psi(0)/RT$, has attained the value of 10 M at a bulk concentration of 10^{-3} M. (The potential at the surface is $\psi(0) = -116$ mv from Fig. 3 and $C(0) = 10^{-3} \cdot \exp 2 \times 116/25.3 = 9.6$ M.) The association constant of 0.1 liter/mole, coupled with the local concentration of 10 M, implies that half of the sites are bound at this concentration (i.e. $\sigma = \sigma^{initial}/1 + K C^{++}(0)$ from equation 4 a). No further binding occurs as the concentration is increased, not because the sites are saturated, but because the concentration of calcium at the interface, $C^{++}(0)$, is independent of the bulk concentration for $10^{-3} < C^{++} < 10^{-1}$ M as shown in Appendix, 3.

phatidylinositol (PI) suspension in a 0.1 M monovalent salt solution, which ceased at a Ca^{++} concentration of about 10^{-3} M. From this type of experiment they deduced an "apparent" association constant of about 10^3 for the binding of Ca^{++} to PI. Although the apparent association constant deduced in this manner is about four orders of magnitude greater than the "intrinsic" association constants calculated here, there is no real discrepancy between the data. According to the Boltzmann equation, the concentration of Ca^{++} at the surface of these charged membranes will be about four orders of magnitude higher than the bulk concentration of 10^{-3} M, and if the release of H^+ is due to Ca^{++} being bound, this binding should only occur below a concentration of 10^{-3} M as the interfacial concentration will remain constant up to a concentration of 10^{-1} M (Appendix, *3.*).

Another experimental approach has been to observe the uptake of radioactive Ca^{++} at the surface of a monolayer formed from phospholipids, as Hauser and Dawson (1967) have done. The films of pure anionic phospholipids they examined gave an adsorption of calcium which was proportional to the total number of net negative charges on the surface. As this technique presumably does not distinguish between Ca^{++} contained in the double layer adjacent to the monolayer and Ca^{++} actually "bound" to the surface of the membrane the results are not inconsistent with our observations on charged lipids. Furthermore, their observation that monovalent ions like Na^+ or K^+ were only effective at displacing Ca^{++} when they were present at a concentration about 10^4 times that of Ca^{++} (10^{-3} vs. 10^{-7} M) agrees with the predictions of double layer theory (see equation 6 *a*).

Our observations with the alkaline earth cations and phosphatidylethanolamine (Fig. 5), those of Abramson et al. (1964 *b*) on phosphatidylcholine, and those of Hauser and Dawson (1967) on both neutral lipids are all in complete agreement and may be summarized by the latter investigators' statement that "no appreciable adsorption occurs unless the lipid . . . bears a net negative charge."

3. *Relevance to Biological Membranes*

The hypothesis under consideration is that the alkaline earth cations may shift the conductance-voltage curves of a nerve along the voltage axis by essentially a screening mechanism. Three necessary conditions for this hypothesis to be tenable are that the diffuse double layer theory be applicable to a charged membrane (see Results section *1.*), that the alkaline earth cations have relatively low association constants with the phospholipids which are major constituents of the biological membrane (see Results section *2.*), and that the membrane adjacent to the sodium and potassium channels in nerve and muscle have a significant negative charge density. The following observations suggest that nerve membranes are indeed negatively charged.

A decrease in the monovalent salt concentration in the interior of a giant axon produces a shift in the steady-state sodium inactivation curves along the voltage axis in the positive direction and this shift, as Chandler et al. (1965) have demonstrated, can be quantitatively explained by assuming that a layer of fixed negative charges exists on the inside of the membrane which may be screened by monovalent ions. The potassium channels are also affected by changes in the internal salt concentration, but to a somewhat lesser degree. The recent observation by Mozhayeva and Naumov (1970) that a decrease in the external monovalent concentration produces little shift in the steady-state conductance-voltage curves of a nodal membrane when the calcium concentration is 2 mM, but does produce a negative shift along the voltage axis when the calcium concentration is about 0.2 mM, is also in complete agreement with the predictions of a screening hypothesis.

An increase in the concentration of divalent ions in the solution bathing a nerve produces a shift in both the sodium and potassium conductance-voltage curves along the voltage axis in the positive direction. This is observed with giant axons from squid (Frankenhaeuser and Hodgkin, 1957), lobster (Blaustein and Goldman, 1968), and cockroach (Narahashi, 1966), with myelinated nerves (Hille, 1968), and with striated muscle fibers (Costantin, 1968). Hille (1968) and Woodhull and Hille (1970), for example, report that at neutral pH a 10-fold increase in the concentration of calcium (2–20 mM) shifts the sodium conductance-voltage curve along the voltage axis by about 21 mv, whereas at pH 10 the shift is 26 mv, very close to the theoretically expected value of 27 mv. At neutral pH, the charge on the membrane is probably not distributed uniformly (Hille, 1970), but an increase in pH is known to neutralize positive amine groups and to increase the magnitude of the negative surface charge density of a bilayer (McLaughlin et al., 1970) or monolayer (Papahadjopoulos, 1968) formed from phospholipids, and presumably produces a high uniform negative surface charge on a biological membrane as well.

We do not wish to suggest that no binding of the alkaline earth cations occurs to nerve and muscle membranes, for although the different alkaline earth cations all produce the same shift for a 10-fold increase in concentration, some are effective at lower concentrations than others (Frankenhaeuser and Hodgkin, 1957; Blaustein and Goldman, 1968). Differences in the effectiveness of the alkaline earth cations are also observed on phospholipid bilayer membranes. It is apparent from Fig. 3 that although Ca and Sr both produce a 27 mv decrease in the surface potential for a 10-fold increase in concentration when $10^{-3} < C^{++} < 10^{-1}$ M, Ca is about 10 times more effective than Sr in reducing the surface potential. This difference was quantitatively accounted for by assuming that calcium could bind to negative sites on the membrane as

well as screening charges. With respect to nerve, this suggestion has also been made by Gilbert and Ehrenstein (1969) and it is interesting that the intrinsic association constant which best explained the shifts produced by calcium on squid axons (0.1 M^{-1}) is exactly the same as the value deduced from the effect of calcium on phosphatidylserine bilayers (Fig. 3). This agreement, however, may be somewhat fortuitous for there is at present no reliable estimate of the surface charge density on nerve (a best fit to their data was obtained with a value of 1 negative charge/120 A^2) and the charge on the nerve is presumably not spread uniformly over the surface. It should also be noted that the argument presented in Appendix, *3.* may be applied to Gilbert and Ehrenstein's theoretical conductance-voltage curve. In the concentration range where the shift along the voltage axis is predicted to be 27 mv for a 10-fold increase in calcium concentration, it is easy to show that the shift is due to calcium screening negative charges rather than binding to them. Furthermore, if the outer surface of the nerve bears a uniform negative charge, divalent ions should lower the magnitude of the negative surface potential, reduce the concentration of permeant monovalent ions at the outer membrane-solution interface, and therefore decrease the magnitude of the conductance. Gilbert and Ehrenstein (1969) did observe a significant decrease in the magnitude of the steady-state potassium conductance of a squid axon bathed in a high potassium solution as the external calcium concentration was increased.

In summary, the above observations provide indirect evidence that a region of the membrane adjacent to both the sodium and potassium channels in nerve bears a net negative charge, as several investigators have previously suggested (e.g., Chandler et al., 1965; Hille, 1968; Gilbert and Ehrenstein, 1969; Mozhayeva and Naumov, 1970). What has not been so widely recognized is that divalent ions will reduce the surface potential, and hence shift the conductance-voltage curves, by essentially a screening mechanism if the charge density is sufficiently high (see, however, the comment on page 469 by Cole, 1968, and the equation used to describe the effects of calcium on nerve by Gilbert and Ehrenstein, 1969). The charge density near the sodium and potassium channels in nerve and muscle need not be as high as that of the phospholipid bilayers, for a density of only one-third the value on these artificial membranes would still produce a potential of about −100 mv at the surface of a membrane bathed in a 0.1 M monovalent ion solution, a potential sufficient to explain the biological effects of the alkaline earth cations in terms of a screening mechanism.

APPENDIX

1. Relevant Equations from the Theory of the Diffuse Double Layer

Integration of the Poisson-Boltzmann equation and application of the appropriate

boundary conditions (e.g. Delehay, 1965), yields the Graham equation:

$$\sigma = \frac{1}{272} \cdot \left[\sum_i C_i \left(\exp \frac{-z_i F \psi(0)}{RT} - 1 \right) \right]^{1/2} \qquad (1\,a)$$

where σ is the surface charge density in electronic charges per square Ångstrom, C_i is the concentration of the ith species in the bulk solution in moles per liter, z_i is its valence, $RT/F = 25.3$ mv at $T = 22°C$, and $\psi(0)$ is the surface potential. If ions of only one valence, z_i, are present, equation 1 a reduces to the familiar Gouy expression

$$\sinh \frac{z F \psi(0)}{2 RT} = \frac{136\sigma}{\sqrt{C}}. \qquad (2\,a)$$

For mixed electrolytes, equation 1 a must be solved numerically and this was done using a standard Newton-Raphson iteration procedure. We also considered the possibility that divalent ions may bind to negative sites on the membrane with an association constant K (in liters per mole):

$$C^{++}(0) + M^- \underset{K}{\rightleftharpoons} CM \qquad K = \frac{CM}{C^{++}(0)M^=} \qquad (3\,a)$$

where C^{++} (0) is the free concentration of divalent ions at the surface of the membrane in moles per liter, M^- is the free concentration of negative sites on the membrane surface, and CM is the concentration of bound ions or neutralized sites. It follows that the charge density, σ, is related to the initial charge density, σ^{initial}, by the expression[3]

$$\sigma = \frac{\sigma^{\text{initial}}}{1 + K\,C^{++}(0)}. \qquad (4\,a)$$

By invoking the Boltzmann relationship we have

$$\sigma = \frac{\sigma^{\text{initial}}}{1 + K\,C^{++} \exp \dfrac{-2F\psi(0)}{RT}}. \qquad (5\,a)$$

Equation 5 a may be combined with equation 1 a to solve for $\psi(0)$ as a function of C^{++} when the initial charge density is known.

2. The Relative Screening Ability of Monovalent and Divalent Ions and the Concept of Ionic Strength

A numerical example of the relative screening abilities of monovalent and divalent ions for a membrane with a high negative surface charge density may be helpful. When only monovalent ions are present ($z = 1$) and the potential is large, $-\psi(0) \gg$

[3] A more complicated expression results if divalent ions bind to single negative sites.

RT/F = 25.3 mv, we can approximate the hyperbolic sine in the Gouy equation 2 a by one-half the exponent and the equation becomes exp $-(F\psi(0)/RT)$ = $(272)^2\sigma^2/C^+$. When only divalent ions are present ($z = 2$) and the potential is large we can write exp $-(F\psi(0)/RT)$ = $-272\sigma/\sqrt{C^{++}}$.

To find the concentration of divalent ions which will produce the same screening effect, or the same reduction in the surface potential, as a given concentration of monovalent ions we equate the potentials and obtain

$$C^{++} = \frac{(C^+)^2}{(272\sigma)^2}.$$ (6 a)

If $\sigma = 1$ negative charge/38 Å^2, a divalent concentration of only $C^{++} = 2 \times 10^{-4}$ M will produce the same screening effect as a monovalent concentration of $C^+ = 10^{-1}$ M.

This difference in the screening abilities of monovalent and divalent ions is much greater than one would anticipate using the concept of ionic strength, $\Gamma \equiv \frac{1}{2}\sum_i C_i z_i^2$, which predicts that divalent ions should have less than 1 % of the screening effect of monovalent ions at the above concentrations. This concept, however, comes from the Debye-Hückel theory (e.g., Robinson and Stokes, 1959, p. 78) where the exponent in the Poisson-Boltzmann equation is linearized, an erroneous assumption when the magnitude of the potential at the surface of a membrane is much higher than 25 mv.

3. The Concentration of Alkaline Earth Cations at the Surface of a Charged Bilayer Membrane is Independent of the Bulk Concentration in the Range $10^{-3} < C^{++} < 10^{-1}$ M.

The numerical solution of the Graham equation 1 a (Figs. 4 and 5) indicates that $\psi(0)$ changes 27 mv for a 10-fold increase in C^{++} in the range $10^{-3} < C^{++} < 10^{-1}$ M (for $C^+ = 10^{-1}$ M, $\sigma^{initial} = 1/38$ Å^2 and $K \leqslant 0.1$ liter/mole). To a good approximation, therefore, the surface potential may be represented analytically by the Gouy expression for divalent ions alone, exp $-(F\psi(0)/RT)$ = $-272\sigma/\sqrt{C^{++}}$, which predicts a 29 (instead of 27) mv change in $\psi(0)$ for a 10-fold change in C^{++}. By combining this expression with the Boltzmann relation, $C^{++}(0)$ = C^{++} exp $-(2F\psi(0)/RT)$, we obtain:

$$C^{++}(0) = (272)^2 \cdot \sigma^2$$ (7 a)

for $10^{-3} < C^{++} < 10^{-1}$ M. The free concentration of divalent ions at the surface of the membrane is thus independent of the bulk concentration, and equations 3 a or 4 a illustrate that the number of bound ions or charge density is therefore also independent of the bulk concentration. This is why the pure screening curve in Figs. 3 and 4 (solid lines) and the curves which incorporate some ion binding (dashed lines) are parallel in this region.

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