J. Membrane Biol. 7, 29–53 (1972)

Comparison of Double Layer Potentials in Lipid Monolayers and Lipid Bilayer Membranes

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Received 7 July 1971

Summary. It is shown that the Gouy-Chapman double layer analysis adequately describes the variation of the surface potential of monolayers of acidic natural lipids over a wide range of surface charge density and salt concentration. It is also shown that the potential which initially appears when an electrolyte gradient is rapidly imposed across a bilayer membrane is due to a difference in the double layer potentials on the two sides of the membrane. This conclusion follows from the fact that the observed bilayer potentials arise much more rapidly than can be accounted for by charge migration across the membrane and from the observation that the bilayer membrane concentration potentials, when measured immediately after establishment of a gradient, are equal to the surface potential change observed when the subphase concentration of a monolayer of the same lipid is changed by an amount equal to the gradient across the bilayer. The bilayer potential and monolayer potential changes, so measured, agree in a number of different electrolyte solutions over a wide range of electrolyte concentrations and surface charge densities. Because of this agreement and the applicability of the Gouy theory to monolayers, initial bilayer potentials may be calculated if the composition of the mixture used to form the membrane is known, provided that the pK's and areas of such components are available. In the absence of this information, membrane potentials may be calculated from electrophoretic data on the membrane lipid mixture; the conditions under which the latter approach is possible have been determined. The experimental results indicate that the composition of monolyers and bilayers spread from the same lipid mixture in decane are very similar, that the composition of the two types of film closely resembles the composition of the solution used to generate them, and that bilayer membranes are close-packed. The evidence further indicates that if any hydrocarbon solvent remains in these bilayers, it must be so situated that it contributes little, if anything, to the surface area. The steady state potential in the bilayer membrane system is frequently not identical with the initial potential which supports the hypothesis that in many cases only a fraction of the electrical conductance of unmodified membranes is caused by the ions which constitute the bulk electrolyte. An expression for the relationship between diffusion and double layer potentials has been derived which shows that, in the absence of any intrinsic selectivity of the hydrocarbon region of the membrane for hydrogen, hydroxyl, or impurity, the two potentials should be identical.

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The lipid bilayer model membrane system has, since its introduction in 1962 (Mueller, Rudin, Tien & Wescott, 1962) enjoyed wide acceptance in the study of a variety of membrane permeability and stability phenomena. Because these membranes are presumed to possess the structure of the lipid core in the model of cellular membranes proposed by Davson and Danielli (1943) and elaborated upon by Robertson (1959), it has been of considerable interest to compare the permeability properties of the model system with those of natural membranes.

Investigations in a large number of laboratories have shown that unmodified bilayer membranes possess resistances which are very high, considerably higher than those exhibited by natural membranes, but not inordinately high for a continuous hydrocarbon sheet. With the aim of determining the ionic selectivity (relative permeability of cations and anions) of these membranes, several investigators have measured the potentials exhibited by bilayers under an electrolyte gradient, and have applied the equation for diffusion potentials to calculate ionic transference numbers. A number of results indicate slight or no selectivity by membranes formed from uncharged lipids (Mueller *et al.*, 1962; Lev & Buzinsky, 1967; Andreoli, Bangham & Tosteson, 1967; Hopfer, Lehninger & Lenarz, 1970) and cation selectivity for membranes formed from negatively charged lipids (Andreoli *et al.*, 1967; Hopfer *et al.*, 1970). Henn, on the other hand, found only slight cation selectivity in membranes formed from the negatively charged phosphatidyl serine (Henn & Thompson, 1969) while Miyamoto and Thompson (1961) observed a small degree of selectivity for cations in membranes formed from the neutral lipid phosphatidyl choline.

Andreoli *et al.* (1967) and Hopfer *et al.* (1969) have pointed out that the surface charge must influence the permeability of these membranes, although they did not consider in detail the mechanism by which this influence is exerted. Hopfer *et al.* have reiterated the point made by Teorell a number of years ago that in the case of charged membranes, there exists a boundary potential at the two surfaces in addition to a diffusion potential across the interior (Teorell, 1953).

This investigation is concerned with the quantitative description of boundary potentials at the interface between a lipid layer and an aqueous solution and with the mechanism by which surface charge affects the ionic selectivity of lipid bilayers. The approach we have used is to isolate the boundary potential effect from the diffusion potential by investigating the surface potentials of monolayers. Monolayer membranes are unique in that the boundary effects can be studied in the absence of transport by the simple device of resolving the bilayer into two monolayers. For this approach

we ascertained that the Gouy-Chapman treatment may be applied to monolayers to describe changes in surface potential with changes of subphase electrolyte concentration. Next, we isolated the boundary potential from the total bilayer potential by methods which allow rapid establishment of an electrolyte gradient; this potential was then compared with that expected from the algebraic sum of surface potentials of two monolayers placed back to back. Finally, an expression was derived which relates the overall transference numbers to the surface charge density of a hypothetical bilayer in which the core material is intrinsically non-selective. Utilizing this treatment it is possible to evaluate the properties of the core of membranes which may indeed be selective.

Materials and Methods

Phosphatidyl choline (PC) was extracted from egg yolks and purified by silicic acid chromatography. Phosphatidic acid (PA) was prepared from it by enzymatic hydrolysis (Papahadjopoulos & Miller, 1967) and purified by silicic acid chromatography. Phosphatidyl serine (PS) was extracted from sheep brain and purified according to Rathbone and Maroney (1963). Whole brain phospholipid extracts were prepared according to Folch (*see* Folch, Lees & Sloane-Stanley, 1957). Cholesterol was recrystallized from absolute alcohol. Decane and tetradecane were freed from polar impurities by passage over alumina. Water was twice distilled, the second time from permanganate. Other chemicals were reagent grade whenever possible.

Monolayer Measurements

Surface potential measurements were made in a small (8 ml) teflon trough. An Americium air electrode was positioned above the trough and a calomel pH reference electrode was inserted into it. The latter electrode was grounded and the air electrode was connected to the input of a Vibron 33B electrometer. The entire apparatus was enclosed in an aluminum box which afforded electrostatic shielding and protection from currents of air. Prior to an experiment, the trough was alternately washed with water and methanol until the surface potential of distilled water read more negative than 350 mV. This criterion was chosen on the basis of experience; changes of surface potential with changes of electrolyte concentration tended to be sluggish and erratic when the initial water surface potentials were more positive than -300 mV. A record of the experiments was obtained from a strip chart recorder connected to the output of the electrometer. Stirring was accomplished with a magnetic flea driven at about 100 rpm.

After filling the trough with salt solution (usually 10^{-3} M), monolayers were spread by the addition to the surface of a decane or tetradecane solution (5 μliters unless otherwise noted) containing 4% phospholipid, 0.05% methanol, and when indicated, 1% cholesterol. The amounts were very much in excess of that necessary to cover the surface with a close-packed monolayer and, to assure that the excess remained at the sides of the trough rather than as lenses in the center of the surface, the hydrocarbon solution was injected against the side of the trough with a microliter syringe.

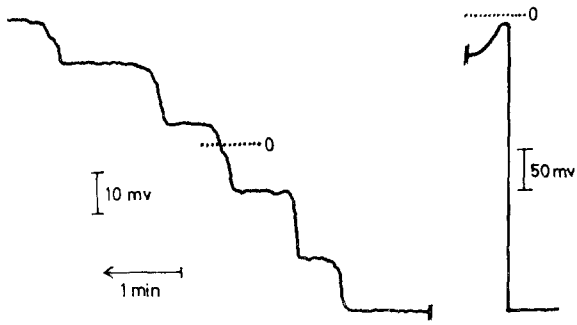

Fig. 1. Dependence on salt concentration of the surface potential of a monolayer of charged lipid. The tracing begins at approximately -350 mV with the surface potential on 10^{-3} M KCl. At the large discontinuity, a monolayer of brain phospholipid and cholesterol was spread from decane solution. The potential rapidly swings positive and then declines slightly as the decane evaporates. The sensitivity was then increased by a factor of 5 (break in tracing) and after approximately 2 min, concentrated KCl was added to bring the subphase concentration to 0.003 M. Subsequent additions of concentrated salt raised the concentration to 0.01, 0.03, and finally to 0.1 M. With each addition of electrolyte the surface potential changes rapidly to a more positive potential

The initial potential observed immediately after adding the lipid solution drifted (negatively) when decane was the solvent but remained constant when tetradecane was the solvent. This drift, which was evidently caused by evaporation of the relatively volatile decane, diminished after 1 to 2 min, and when the potential had become constant to within 2 mV per min, injections of concentrated electrolyte (usually 2 M) were made through the monolayer with a microliter syringe. The new potential became constant in about 15 to 30 sec after the addition; successive additions of electrolyte were made at 1- to 2-min intervals and in this manner a record of the change of surface potential with salt concentration from 10^{-3} to 10^{-1} M was obtained. A typical record of such an experiment is shown in Fig. 1.

Bilayer Measurements

Membranes were formed from the same lipid solution which was used for surface potential measurements described above. Membranes were formed on the end of a polyethylene tube which projected into a simple acrylic chamber. The latter, known as the front compartment, was provided with an inlet and outlet so that the contents of the compartment could be changed rapidly. The volume of the front chamber was sufficiently small so that its contents could be completely changed in less than 20 sec. The polyethylene tube, which comprised the back chamber was connected to a calomel electrode and to a microliter syringe. The syringe allowed for adjusting the membrane to planarity.

For resistance measurements, a known potential was impressed across the membrane via a calomel electrode inserted in the front compartment. The electrode from the back compartment could be switched into one of a series of resistors, and the potential drop across the resistor, as measured with the Vibron electrometer, was used to calculate the membrane resistance.

For potential measurements, the front electrode was grounded and the back electrode switched directly into the input of the electrometer. The output of the latter was fed into a strip chart recorder.

Membranes were usually formed in 10^{-3} M electrolyte solution and the front compartment was replaced with 0.033 M solution. When potential had reached its maximum value, the 10^{-3} M solution was returned to the front compartment. The same solutions were alternated two more times and the average of the three potentials was taken as the concentration potential for that membrane. The process was repeated for concentrations of 0.01, 0.033, and 0.1 M.

Occasionally, potentials were measured in a simple cell having two open compartments separated by a partition. After forming the membrane in a low salt solution, a small volume of concentrated solution was injected into the bottom of the grounded compartment with a microliter syringe. The solution was then vigorously stirred with a magnetic flea. In this way, the concentration could be changed in less than 5 sec. In those instances where both methods were applied to membranes having the same composition, the results agreed to within 10%.

Membrane resistance was routinely checked before and after each experiment to ascertain that the resistance was not so low and the time constant consequently so short as to preclude measurement of the development of the boundary potential separately from that of the diffusion potential. Membranes with resistances below about 100 MΩ cm^2 were rejected.

Electrophoretic Measurements

Microelectrophoresis was carried out as described by Bangham, Flemans, Heard, and Seaman (1958). The same lipid solution that was used for forming bilayers and monolayers was dispersed in the appropriate aqueous phase by sonication of the mixture for 30 sec in a small cleaning bath sonicator. The ratio of lipid solution to aqueous solution was the same as that used in the monolayer experiments, i.e., 5 μliters lipid solution to 8 ml aqueous phase.

Theory, Results, and Discussion

I. *Relationship Between Surface Charge Density, Surface Potential, and Electrolyte Concentration in Lipid Monolayers*

As Davies has shown, highly charged monolayers give rise to surface potentials which are a function of the electrolyte concentration in the aqueous phase (Davies & Rideal, 1963). For such high surface charge densities, the change in potential is the thermodynamic maximum, i.e., 58 mV (at 20 °C) per 10-fold change in subphase electrolyte concentration. In the following discussion, the major departure from Davies' reasoning is that here we shall consider the consequences of low as well as high charge densities.

The Gouy equation may be written

$$\varphi_0 = \frac{2RT}{F} \sinh^{-1} \frac{\sigma}{c^{\frac{1}{2}}} \left(\frac{500\pi}{DRT}\right)^{\frac{1}{2}} \tag{1}$$

where σ is the surface charge density, c the electrolyte concentration in the subphase, D its dielectric constant, φ_0 the Gouy potential at the plane of surface charges, and F, R and T have their usual meaning. The general

relationship between potential change and concentration change can be obtained by simply taking the derivative of Eq. (1) with respect to c. For present purposes, it is more useful to have the potential variation with the logarithm of the concentration change. The appropriate derivative is in this case (assuming σ to be constant),

$$\frac{d\varphi_0}{d(\ln c)} = -\frac{RT}{F}\tanh\frac{\varphi_0 F}{2RT}. \tag{2}$$

This expression may, for small changes in φ and c, be written

$$\Delta\varphi_0 = -\frac{RT}{F}\ln\frac{c_1}{c_2}\left[\tanh\frac{\varphi_0 F}{2RT}\right] \tag{3}$$

and is seen to be the form of the familiar Nernst equation modified by the bracketed term on the right.

Since the hyperbolic tangent has a range from 0 to 1, it is clear from Eq. (2) that a plot of φ_0 *vs.* log c will give a slope dependent upon the value of φ_0, varying from 0 to 58 mV per decade change in concentration at 20 °C.

Now, the hyperbolic tangent rapidly approaches its upper limit of 1 and to within 5%, the upper limit is reached at tanh 2. Since $F/2RT$ is 52 mV, it is clear that at a φ_0 of 100 mV, the right-hand sides of Eqs. (2) and (3) will closely approximate that of the Nernst equation. The surface potential φ_0 is, by Eq. (1), dependent both upon surface charge density and electrolyte concentration; so a Nernst slope is expected for moderate surface charge densities in low salt concentration or for high surface charge densities in all but extremely high salt concentrations. The latter case was investigated by Davies who indeed found a 59 mV slope per decade charge in electrolyte concentration at 25 °C.

It should be recognized that the above discussion considers only *changes* in surface potential with changes in electrolyte concentration. The total surface potential, as measured with an ionizing or vibrating reed electrode will also measure contributions from the vertical component of any permanent dipole moments of the molecules of the monolayer. These will generally be considerably larger (300 to 500 mV) than the changes produced by changes in salt concentration (max 58 mV per 10 × change). It is assumed that the change in surface potential produced by a change in salt concentration is attributable entirely to the shift in the space charge of the counterions with respect to the fixed charges in the surface, i.e., the orientation of the permanent dipoles of the monolayer are taken to be independent of the electrolyte concentration of the sub-phase. This will probably be true if the monolayer is maintained at constant area so that the molecular packing remains invariant.

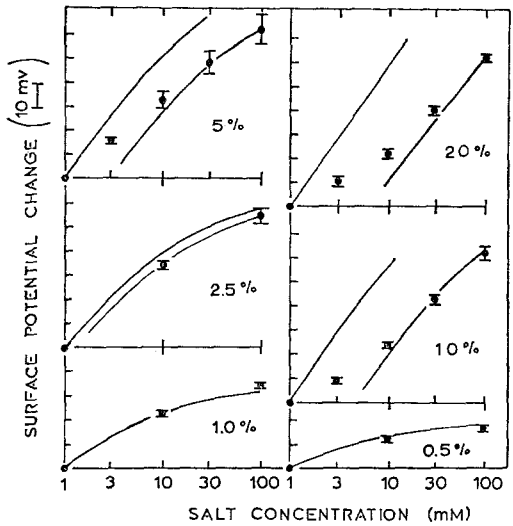

Fig. 2. Comparison of the concentration dependence of surface potentials with predictions of the Gouy equation. Surface potentials of monolayers of lecithin containing various percentages of PA were determined as a function of KCl concentration in the subphase. The lines are calculated from Eq. (1) assuming an area per molecule of 62 Å^2. The experimental values contain an unknown additive factor which arises from permanent dipoles and in the figure, experiment and theory have been chosen to coincide at the highest and lowest concentrations

The accuracy of Eqs. (1)–(3) was checked using mixtures of PC and PA having molar ratios ranging from 200:1 to 5:1. The PA had been prepared from PC by enzymatic hydrolysis, so the lipid composition and the limiting surface area of the two molecules were the same. PA is completely ionized at the pH of the distilled water used for the experiments, so the charge density of monolayers formed from these mixtures is readily calculated. The area per molecule at collapse pressure used in the calculation of charge densities was 62 Å^2 (Bernard, 1958). The change of pH of the subphase accompanying the spreading of monolayers did not exceed 0.3 pH unit.

Fig. 2 shows the change in surface potential for PA-PC monolayers as the electrolyte (KCl) concentration is changed from 10^{-3} to 10^{-1} M. The data are compared to the potential change calculated from Eq. (3). Because the potential of the permanent dipoles of the monolayer makes an unknown contribution to the total potential, it is not possible to determine at which (if any) concentrations the theoretical and experimental curves should coincide. Except for the monolayers with very low charge densities, the agreement is better for differences from the highest concentration. The discrepancy appears to lie in too small a slope in the dilute solution.

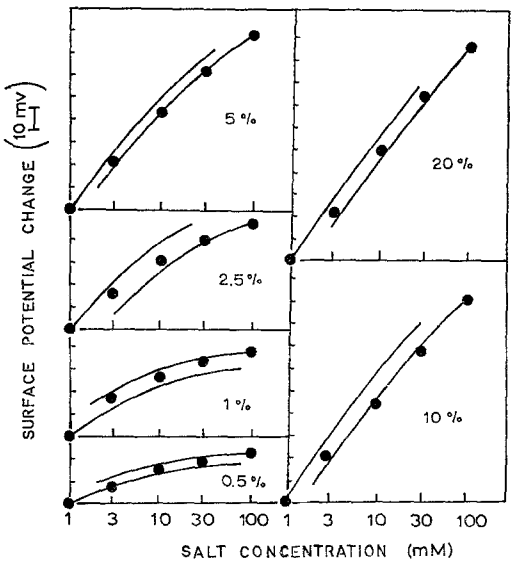

Fig. 3. Comparison of the concentration dependence of surface potentials with predictions of the Gouy equation. Chelating agent present in the subphase. Conditions were as described in the legend for Fig. 2 except that initially the subphase contained 9.9×10^{-4} M KCl, 1×10^{-5} M Na^+ (total cation $= 10^{-3}$ M) and 2.5×10^{-5} M EDTA

This is the kind of discrepancy which would result if there were a low concentration of multivalent ions already in the solution when the KCl was added, since di- or trivalent ions produce space charges equivalent to that of univalent ions at much lower concentrations. The phosphatidic acid had been prepared in a high concentration of Ca^{++}, so some contamination by the latter was not an unlikely possibility. To check this, the experiment was repeated with an EDTA solution as the subphase. In this case, the total univalent cation concentration was 10^{-3} M, the EDTA concentration 2.5×10^{-5} M (Na salt) and the pH 6.16. (In separate experiments it was found that increases in EDTA concentration produced no further changes in slope.)

The data from these experiments are plotted in Fig. 3. Again they are plotted to coincide with the theoretical curves at either the highest or the lowest concentration. The most notable difference in Figs. 2 and 3 is that EDTA eliminates the diminished slope at low concentrations and the potential change for the range of concentrations is considerably increased. These differences are in accord with the suggestion that in the absence of EDTA, multivalent ions compete with the K^+ ions.

The agreement between the Gouy equation and the data is not as good when the data are normalized to zero at 10^{-3} M as it is when they are normalized to zero at 10^{-1} M. Although the reason for the discrepancy is not clear, the most obvious possibility is that the permanent dipole moment is not strictly independent of concentration. There are a number of deficiencies in the Gouy theory (Haydon, 1964) but it would be necessary to use a better defined system than the present for an accurate experimental determination of the magnitude and direction of its inadequacies. For purposes of approximately calculating boundary potential contributions to bilayer potentials, the theory should be adequate.

II. *Double Layer Potentials in Bilayer Membranes*

Let us now consider the consequences of placing two charged monolayers back to back to form a bilayer membrane separating two aqueous phases. Electrodes placed in the aqueous phases will measure the algebraic sum of the two Gouy potentials and the diffusion potential across the membrane proper. If we now consider a change in the concentration on one side of the membrane, it will be obvious that the boundary potential will adjust as rapidly as the surface and bulk electrolyte phases equilibrate. The development of the diffusion potential on the other hand, is limited by the rate at which net charge can migrate across the membrane. This process is dependent upon the magnitude of the difference between t^+ and t^- and the absolute value of the ionic conductivities. Although it is generally not possible to calculate the kinetics of a diffusion potential development without information on the properties of the interior of the membrane, it is obvious that the diffusion potential build-up cannot exceed the charging rate for a RC network having the time constant of the membrane. This is of the order of 2 or more min for the membranes studied here, however, so for changes of potentials across bilayers which are measured in the first 10 sec or so following a concentration change, the contribution of the diffusion potential will be negligible.

To avoid the complication of a diffusion potential, we presume for the present that no current flows across the membrane. As noted, in practice such a condition is attainable by measuring the potential immediately after the electrolyte concentration has been changed.

If, as we have assumed, the permanent dipoles are independent of salt concentration, the field due to the dipoles will yield a resultant of zero regardless of any changes of electrolyte on either side of the membrane. On the other hand, the Gouy potential at the two surfaces will depend upon

the salt concentration in the aqueous phases adjacent to these surfaces. When a salt gradient is imposed across the membrane, the two surface potentials will differ and their algebraic sum will be non-zero. If the electrolyte concentration on one side of the membrane (*b*) is held constant at c_1, while that on the other side (*a*) is changed to c_2, the membrane potential (as measured with KCl-bridge electrodes) will be

$$E = \Delta\varphi_a - \Delta\varphi_b, \tag{4}$$

and from Eq. (3),

$$E = -\frac{RT}{F}\left(\ln\frac{c_1}{c_2}\right)\tanh\frac{\varphi_0 F}{2RT} + \frac{RT}{F}\left(\ln\frac{c_1}{c_1}\right)\tanh\frac{\varphi_0 F}{2RT} \tag{5}$$

or,

$$E = -\frac{RT}{F}\left(\ln\frac{c_1}{c_2}\right)\tanh\frac{\varphi_0 F}{2RT}. \tag{6}$$

Likewise, if small changes in concentration are considered, the slope of membrane potential *vs.* ln *c* becomes, from Eq. (2),

$$\frac{dE}{d(\ln c)} = -\frac{RT}{F}\tanh\frac{\varphi_0 F}{2RT}. \tag{7}$$

The only difference between the situations described by Eqs. (2) and (7) is that in the initial state of the latter case, the second monolayer provides a "buck out" to that of the first monolayer, and changes are measured from zero.

III. *Comparison of the Initial Bilayer Membrane Potential with the Surface Potential of a Monolayer of the Same Lipid*

Although the surface potential results showed that the Gouy equation gave an adequate description of monolayers, the possibility had to be considered that one monolayer surface of a bilayer membrane may not, as far as the electrical double layer is concerned, be identical to a compressed monolayer at the air-water interface. If the area per charged molecule in the two types of monolayers were different, then the boundary potentials would also have to be different. There are two possible ways in which the area per charge could differ in the two systems. The first, which would apply to mixed lipids, is that the composition of the monolayers could differ, and the second is that the molecular packing could differ, i.e., one might be more compressed than the other.

Comparison of the concentration dependence of the double layer potential in monolayers and bilayers has been made in two ways: (1) with

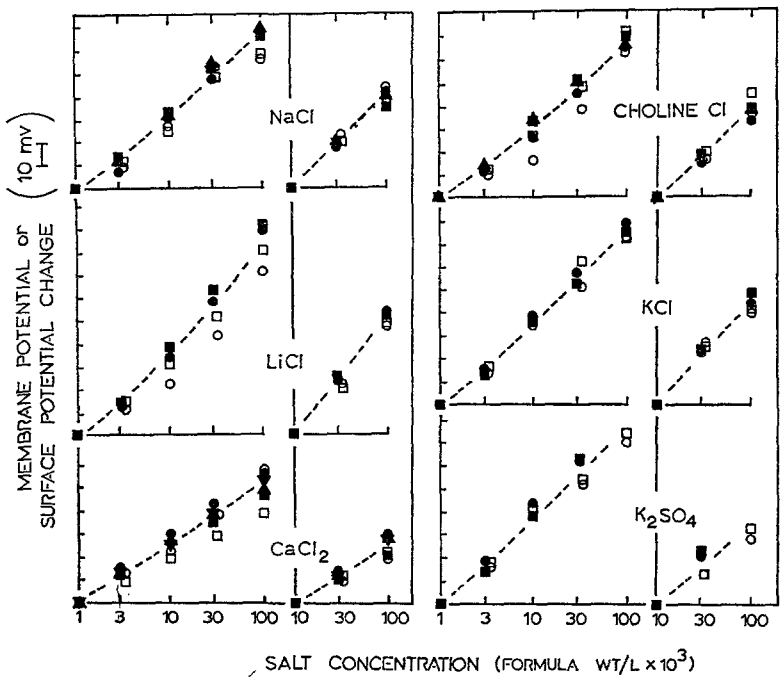

Fig. 4. Comparison of initial bilayer membrane concentration potentials with monolayer potential changes in brain phospholipid system. Initial salt concentration for the bilayer and monolayer experiments was 10^{-3} formula wt/liter. The concentration of salt on one side of the bilayer and in the subphase of the monolayer was increased in the increments shown on the horizontal axis. The resulting potential changes are shown on the vertical axis. Monolayer data are represented by filled symbols (duplicate-quadruplicate) and bilayer experiments are represented by open symbols (in duplicate). The changes in concentration were the same for the two systems except at the second and fourth steps where the concentrations in the bilayer experiments were $3^1/_3$% higher than those of the corresponding monolayer experiments. On the right, the data for each electrolyte are replotted to show the changes in the second decade, normalized to zero at 10^{-2} F

constant lipid composition and variable electrolyte; and (2) with constant electrolyte and variable lipid composition.

For the experiments in which the lipid mixture was constant, the mixture chosen consisted of 4% whole brain phospholipids and 1% cholesterol in decane. Since brain phospholipids are a complex mixture, this choice seemed to be most promising for detection of differences between the composition of the two systems. Monolayer data were obtained in the same manner as those of the previous section. Bilayer data were obtained as described in Materials and Methods. Potential changes over two decades of concentration were measured using the chloride salts of Li, Na, K, Ca, and choline, and the sulfate salt of K. The potential of the lowest concentra-

tion (10^{-3} M) was arbitrarily taken as zero in the case of monolayers and the other potentials plotted as differences from this value. Since the bilayers were formed in 10^{-3} M solutions, the initial potential was usually very close to zero. The results of these experiments are shown in Fig. 4. On the right-hand side of the section for each electrolyte, the potential changes for the decade $10^{-2} - 10^{-1}$ M are plotted. These values were calculated by subtracting the potential at 10^{-2} M from those at the two higher concentrations. The data in the latter plot are more tightly grouped because most of the differences between duplicate or triplicate experiments are confined to the first decade. It is apparent that, for all six electrolytes, there is little difference between the boundary potential changes in the monolayer and the initial bilayer potentials. This strongly suggests that the packing and composition of the lipids in the two systems are, if not identical, very similar.

For the second comparison the charge density was varied using PS-PC mixtures. PS was suitable for these experiments because it forms stable, high-resistance bilayer membranes. The initial potentials developed across bilayers of PS-PC in various proportions under gradients of 1:10 mM and 1:100 mM KCl are shown in Fig. 5. The change in the surface potential of monolayers formed from the same lipid mixtures as subphase concentration increased from 1 to 100 mM KCl is shown in Fig. 5. Also shown in Fig. 5 is the change in the surface potential of monolayers formed from the same lipid mixtures as subphase concentration increased from 1 to 100 mM KCl. As in previous experiments, the data are normalized to zero at 1 mM. Except for the lowest concentrations of PS in PC, where experimental error is likely to be the largest, the monolayer and bilayer potential variations with electrolyte concentration are very much the same.

Since the solutions were unbuffered and because the pH was close to one of the pK's of the PS, the surface charge density of the mono- and bilayers cannot be computed with any accuracy.

Several important points emerge from the fact that there is approximate agreement between monolayer and bilayer potentials and both of these with potential changes predicted by the Gouy theory. First, it appears likely that the composition of bilayer membranes, at least for a simple mixture of a charged and an uncharged phospholipid, cannot be grossly different from the lipid composition of a monolayer spread from the same solution mixture. This is perhaps to be expected, since the film formation in the two systems proceeds through similar steps: generation of an oil-water interface, migration of the amphiphile to the interface, and retraction of the bulk oil phase into lenses or a torus.

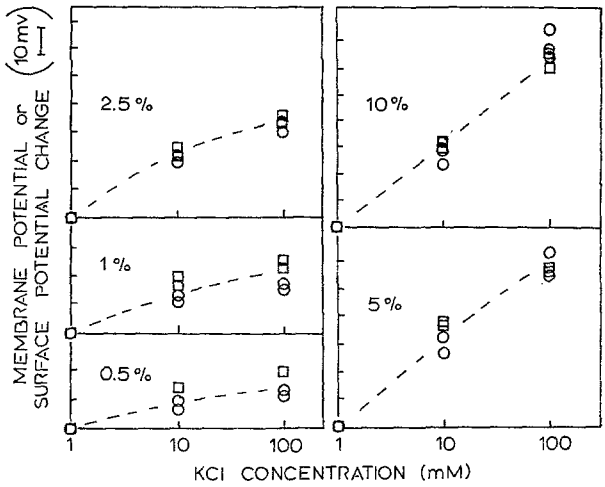

Fig. 5. Comparison of initial bilayer membrane concentration potentials with monolayer surface potential changes: PC-PS system. Conditions were as described in the legend for Fig. 4 except that the electrolyte was KCl throughout and the lipid consisted of mixtures of PC and PS. The percentage shown refers to the latter component. Monolayer data are represented by circles and bilayer data by squares

Another approach to the composition of bilayer membranes has been investigated in Haydon's laboratory (Cook, Redwood, Taylor & Haydon, 1968). For soluble surfactants such as glycerol mono-oleate, the method is particularly simple and elegant. It is shown that when the difference between the surface tension of the bilayer and the interfacial tensions of the bulk lipid from which the bilayer was generated is negligible, then the surface excess of the surfactant in the two films is the same. The surface excess can then be obtained from determinations of the interfacial tension between the bulk lipid phase and the appropriate aqueous phase by application of the Gibbs equation. For insoluble surfactants such as PC, however, the Gibbs equation does not apply and they resort to a direct measurement of the amount of phospholipid that is necessary to lower the tension at the oil-water interface to that corresponding to the bilayer. This approach is limited to a single phospholipid component. The present method is, of course, limited to charge density determination.

Henn and Thompson (1968) attempted to determine bilayer composition using radioactive lipids, but the results are ambiguous because of the presence of lenses of bulk lipid solution which remain trapped in the bilayer. The method has also been criticized on theoretical grounds by Haydon (1969) who suggests that the process of isolating the bilayer may lead to changes in its composition.

The present results suggest that, at least when molecules as similar as the phosphatides are employed, the composition of the bilayer is much the same as the phospholipid composition of the solution from which the bilayer is formed. When dissimilar substances such as cholesterol and phospholipids are considered, this may or may not be true. According to the results of one group (Cook *et al.*, 1968), it is not, but according to another (Henn and Thompson, 1968), it is. Probably neither of the methods utilized by these workers is adequate to provide a definitive answer. The question of how much hydrocarbon remains in the bilayer has also been considered by these two groups and both have concluded that at least some of the solvent remains in the membrane. Since we find the charge density of bilayers to be much the same as that of the corresponding monolayer at the air-water interface (from which the decane has evaporated), it is impossible that the decane, if it persists in the bilayer, can contribute appreciably to the membrane area. This conclusion is consistent with the data of Cook *et al.* (1968), since their determination of the area per molecule of PC in a bilayer is nearly identical to the close-packed area of this molecule. Unless the residual hydrocarbon remains in the center of the bilayer, it must therefore occupy voids in lateral positions between the phospholipid molecules. Since it is now generally agreed that a combination of thermal motion and kink(s) in unsaturated fatty acids requires the existence of an unoccupied volume immediately behind the polar group, it seems reasonable to suggest that this is where the hydrocarbon solvent remains.

IV. *Relationship Between Zeta Potentials and Concentration Dependence of Surface Potentials*

Because the concentration dependence of bilayer and monolayer potentials frequently cannot be directly calculated, the possibility of utilizing electrophoretic data for experimentally assessing the surface charge density was explored.

As is well known, the relationship between surface charge density and electrophoretic mobility becomes progressively more difficult to calculate as the charge density increases. It was therefore of value to determine, for a phospholipid system, the range of charge density over which electrophoresis would be useful. This was done by carrying out micro-electrophoresis on mixtures of PC and PA. Varying amounts of PA were mixed with PC. The mixture was then dissolved in decane to a total concentration of 4% (wt/vol). The hydrocarbon was emulsified into 0.001 M KCl, and the

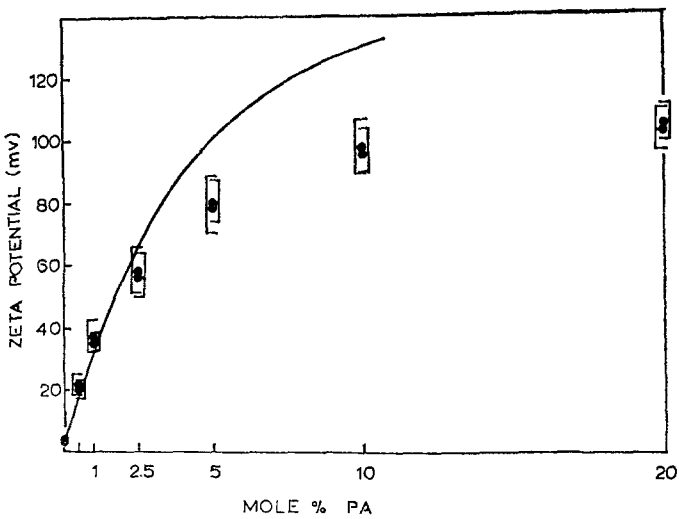

Fig. 6. Zeta potentials of decane droplets containing PC and PA compared with calculated surface potential. Decane solutions of PC with various percentages of PA were dispersed in 10^{-3} M KCl and electrophoretic mobilities of the dispersion measured as described in text. Bars represent standard deviation for each of the duplicate measurements. The line is the surface potential calculated from Eq. (1) assuming an area per molecule of 62 Å^2

zeta potential of the droplets calculated from their electrophoretic mobility according to

$$\zeta = \frac{4\pi\eta\omega}{D} \tag{8}$$

where η and D are the viscosity and dielectric constants, respectively, of water, and ω is the electrophoretic mobility in cm^2/sec/volt. The results are shown in Fig. 6. The curve is the surface potential calculated from the surface charge density (based on an area of PA of 62 Å^2) according to Eq. (1), and the circles are the experimentally determined zeta potentials. As may be seen, the agreement is satisfactory up to about 60 mV.

Electrophoretic mobility data may be utilized for monolayer or bilayer potential measurements in two ways. One is to calculate surface charge density from the zeta potential and then proceed as described in the first section. A second, simpler method is to replace the surface potential by the zeta potential on the right-hand side of Eq. (2), the results being

$$\frac{d\varphi_0}{d(\ln c)} = -\frac{RT}{F}\tanh\frac{\zeta F}{2RT}. \tag{9}$$

This equation gives, for low zeta potentials, the variation of the double layer potential at the particular concentration at which the zeta potential

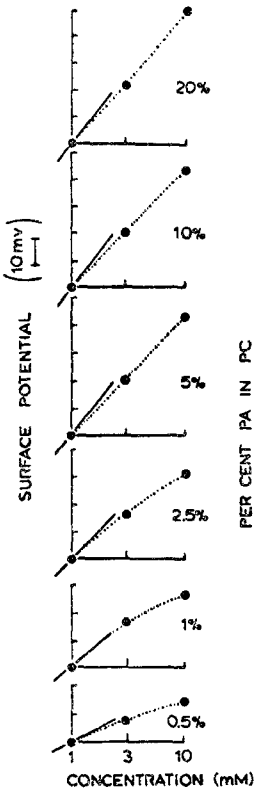

Fig. 7. Comparison of the concentration dependence of the surface potential with that calculated from the zeta potential. The points represent the data for the first decade concentration change of Fig. 3. The lines, representing the concentration dependence of the surface potential at 10^{-3} M, were calculated by inserting the values of the zeta potentials from Fig. 6 into Eq. (9)

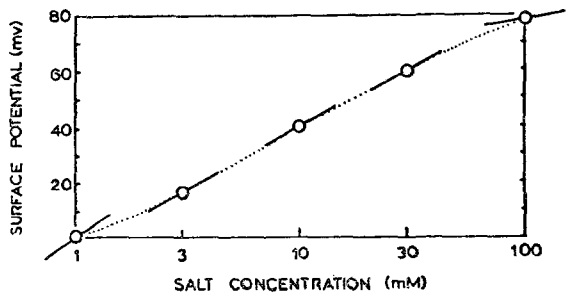

Fig. 8. Calculation for a complex lipid mixture of the slope of the surface potential *vs.* salt concentration using zeta potentials of the lipid dispersion. The circles represent the change with subphase electrolyte concentration (KCl) of a brain lipid monolayer potential. The lines represent the slope of that curve at each particular concentration as determined by applying Eq. (9) to the zeta potentials at those concentrations. Zeta potentials were obtained by electrophoresis of a dispersion of the same decane-brain lipid solution which was used to spread the monolayer

was measured. To test the utility of this equation, we have used the electrophoretic data from Fig. 6 to calculate $d\varphi/d(\ln c)$ at 0.001 M electrolyte concentration. Since the change in potential of monolayers containing

various proportions of PA had been obtained for concentration changes from 0.001 M and higher (Fig. 3), it was possible to compare the values of $d\varphi_0/d(\ln c)$ calculated from electrophoresis with the experimentally determined variation of φ_0 with $\ln c$. Such a comparison is shown in Fig. 7, where the solid line is calculated and the points are the data for the first decade concentration change from Fig. 3. The agreement, which is by no means perfect, is acceptable, considering the fact that surface potential changes at lower concentrations tend to be smaller and therefore less reliable than those at higher concentrations.

The change in boundary potentials with subphase concentration of monolayers of unknown composition is, of course, the situation of greatest interest; therefore, the applicability of Eq. (2) to the mixed lipid system was tested. As described above, a decane solution of 4% brain phospholipid −1% cholesterol was dispersed in solutions of varying concentrations of KCl. The zeta potential in each concentration was determined electrophoretically, and $d\varphi_0/d(\ln c)$ calculated from Eq. (9). The slope was then compared with that obtained from the surface potential-ln c curves obtained from a monolayer of the same lipid solution. The results are shown in Fig. 8, where the short full lines are the slope at each concentration as calculated from electrophoretic data, and the circles represent the monolayer potential data. The dotted lines merely connect the latter data points. The calculated slope agrees well with that of the curve drawn through the experimental points in the middle of the concentration range, but deviates considerably at the extremes. The reason for the smaller predicted slope at higher concentrations is probably because here the double layer is extremely compressed, a significant proportion of the counter ions are within the shear layer, and the zeta potential is consequently underestimated. The deviation at the lower concentration is caused by too small an increase in surface potential with salt concentration. This resembles the behavior of PA-PC monolayers in the absence of EDTA even though EDTA was included in the subphase for these experiments. It is possible that the brain lipid mixture is more heavily contaminated with multivalent ions than is PA and the amount of EDTA was simply insufficient to chelate it all. Effects of higher concentrations of EDTA were not determined for the brain lipid system.

Zeta potentials of brain lipid dispersions indicate that these mixtures contain the equivalent of almost 30% of a singly charged component. Reference to Fig. 6 reveals that at such charge densities, the divergence between zeta and surface potentials becomes very considerable. In view of this divergence, it is appropriate to ask why the agreement between the

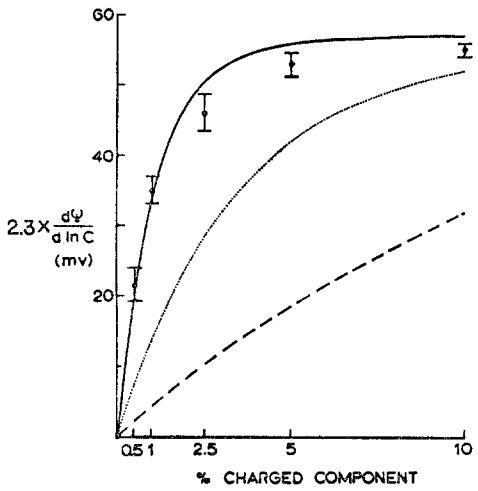

Fig. 9

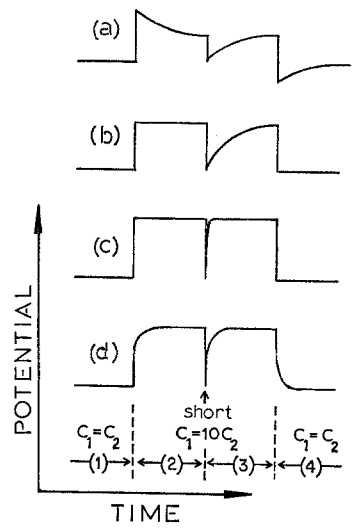

Fig. 10

Fig. 9. Dependence upon surface charge density of the derivative of surface potential with respect to logarithm of subphase concentration. The full line, calculated from Eq. (2), describes how the concentration dependence of the surface approaches a Nernst slope at 10^{-3} M monovalent electrolyte concentration with a small percentage charged component (at 62 Å/charge). The approach to a Nernst slope is much less rapid at 10^{-2} M (dotted line) and 10^{-1} M electrolyte. The points and error bars were calculated from the average values and standard deviations, respectively, of the zeta potentials of Fig. 6 using Eq. (9) in terms of common logarithms

Fig. 10. Diagrammatic representation of various observed time dependencies of bilayer membrane concentration potentials. During time periods (1) and (4), the concentration on both sides of the membrane are equal. During (2) and (3), a concentration gradient exists across the membrane. During (3) the potential is recovering from short-circuiting at the end of (2). For the explanation of the different behavior represented in (*a*) through (*d*), *see* the text

two sets of data of Figs. 7 and 8 is not very much worse. By substituting in the right-hand side of Eqs. (2) or (9) the value of the surface or zeta potential, in terms of surface charge density from Eq. (1), and then assuming an area per charged component, one can calculate the relationship between $d\varphi/d(\ln c)$ and the percentage charged component. This has been done for an area per charge of 62 Å^2 (that of close-packed lecithin) and the results presented in Fig. 9. It may be seen that the value of $d\varphi_0/d(\ln c)$ rapidly approaches the limit of 58 mV per decade, being 56 mV at as low a concentration as 5%. Also included in the figure are the values of $d\varphi_0/d(\ln c)$ calculated from the actual values of the zeta potentials shown in Fig. 6

(measured at 0.001 M). It may be seen that the theoretical potential *vs.* ln concentration slopes, rather than diverging from those calculated from the zeta potential, actually come into better agreement at high charge density. This behavior is simply a consequence of the fact that $\tanh(\varphi_0 F/2RT)$ is close to the limit of unity at potentials above about 80 mV. This means that the variation of the double layer potential with concentration for close-packed monolayers will be close to the thermodynamic limit for the vast majority of lipids of natural membranes. This will, of course, be less true as the electrolyte concentration increases, and curves for 0.01 M (dotted) and 0.1 M (dashed) have been included in Fig. 9 to illustrate this effect. At 0.1 M the slope does not exceed 50 mV per decade until 25% charged component is reached.

V. *Relationship Between Surface Charge Density and Diffusion Potential of Bilayer Membranes*

The potential which appears across highly charged bilayer membranes when a salt gradient is suddenly imposed on the membrane may or may not remain constant. We have observed four different types of time dependent behavior. These are illustrated diagrammatically as (*a*), (*b*), (*c*) and (*d*) in Fig. 10, where the potential is recorded as a function of time while four operations are performed. Initially, (1) the membrane has equal salt concentration on both sides. Next, (2) the salt concentration on one side is instantly raised or lowered by a factor of 10. Then, (3) the electrodes from the two compartments are shorted together. Finally, (4) the solution on one side is returned to the original concentration. In (*a*) the most common behavior is illustrated. At the change of concentration, a potential quickly appears. This is the double layer potential which, as described above, is in good agreement with predictions of the Gouy equation. This potential slowly decays to some intermediate value, which after short circuiting, builds up again very slowly. When the concentrations are finally returned to equality, the potential overshoots and slowly returns to zero. Less frequently, the behavior depicted in (*b*) is observed. Here the potential rises rapidly when the salt concentration is changed, remains approximately constant, but only slowly regenerates after shorting. In (*c*) the characteristic feature is a constant potential and a rapid regeneration after shorting. This feature is also observed in (*d*); however, following the initial appearance of the double layer potential, the potential continues to rise to some higher value.

In each of these cases the behavior of the membrane potential is a function of the contributions of the double layer potentials at the surfaces

of the membrane, a diffusion potential across the membrane proper, and the time constant for the development of each of these potentials.

With the possible exception of case (*c*), the initial potential can be unambiguously ascribed to a change in boundary potential as described in Section II. above. Whether or not this decays depends either upon the degree of permselectivity of the membrane, or the presence of permeable charge carriers other than the ions of which the concentration gradient is constituted. The diffusion potential across the membrane as a whole is described by the equation

$$E_D = \frac{RT}{F}(2t^+ - 1)\ln\frac{c_1}{c_2} \tag{10}$$

where c_1 and c_2 are the salt concentrations in the bulk aqueous phases and t^+ is the positive ion transference number for the membrane as a whole. Now, if we take the difference between c_1 and c_2 to be within the range where Eq. (6) approximately holds, we can, by combining equations, obtain

$$\frac{2t^+ - 1}{\tanh\frac{\varphi_0 F}{RT}} = \frac{\Delta\varphi_0}{E_D}. \tag{11}$$

From this equation it is clear that the boundary potential difference will equal the diffusion potential only if t^+ is such that

$$2t^+ = \tanh\frac{F\varphi_0}{2RT} + 1. \tag{12}$$

If this is true, no charge transfer will occur across the membrane when the salt concentration on one side is changed. This follows because the driving force due to the concentration difference between the two surfaces of the membrane is exactly balanced by the potential in this type of situation. If, on the other hand, the equality does not hold, then these forces will not balance; when t^+ is larger than dictated by Eq. (12) the gradient of concentration exceeds the gradient of voltage and positive ions will diffuse from the higher to the lower concentration, and if t^+ is lower than prescribed by Eq. (1), the reverse will be true and negative ions will diffuse in the same direction. The end result in both cases is a potential which satisfies Eq. (10).

The situation is somewhat similar when the electrodes are short-circuited, except that in this case there is no initial potential and regeneration of the potential is purely a diffusion process. As noted above, for a high resistance bilayer it is a slow process.

A second reason for the change of membrane potential shown in Fig. 10*a* could be that there are other permeable ions in the system besides those

which constitute c_1 and c_2. If this is true, the membrane potential will be described, at least approximately, by the Goldman-Hodgkin-Katz equation:

$$E = -\frac{RT}{F} \ln \frac{P_K(K_1) + P_{Cl}(Cl_2) + \sum P_C(C_1) + \sum P_A(A_2)}{P_K(K_2) + P_{Cl}(Cl_1) + \sum P_C(C_2) + \sum P_A(A_1)} \tag{13}$$

where the P's are permeability coefficients and the summations are taken over all cations (C) and anions (A) other than K and Cl. The other permeable ions could be H^+ or OH^-, or impurities in either the aqueous or membrane phases. If either OH^- or H^+ have appreciable permeabilities, or if there are charged impurities in the membrane, the initial membrane potential must decline, since the conductance of these ions will act as a shunt across the membrane. On the other hand, impurities in the aqueous phase could cause either an increase or a decrease in the potential, depending upon whether the most permeable substance was cationic or anionic.

The reason for the overshoot sometimes observed when the gradient across the membrane is returned to zero (step 4 in Fig. 10) is again due to the change in the double layer potential. That this is the case can be demonstrated by shorting the membrane and then, before diffusion causes regeneration of the potential, returning the gradient to zero. A potential is seen which, in high resistance membranes, is always equal and opposite to the initial potential produced when the gradient is imposed. This potential then decays to zero with the time constant of the membrane.

On the basis of the above considerations, the behavior of the membranes depicted in Fig. 10 can be analyzed. Case (*b*) is a high-resistance membrane in which the only ions contributing appreciably to the conductance are K^+ and Cl^-. The latter is also true of (*c*), but this membrane has a low resistance, probably because of a leak. Since the conductance of the system is high, the diffusion potential is rapidly established, and the double layer potential change cannot be temporally separated from the diffusion potential. Case (*d*) was seen only with membranes which had slight leaks, i.e., their resistances were lower than normal, but not as low as the examples depicted in (*c*). Here there is a small leak pathway which is more selective for positive ions than predicted by Eq. (12). The leak in this case is probably between the support and the membrane where a negatively charged channel may exist. Case (*a*), the most common situation, requires a more detailed analysis.

As noted, the decline of the initial boundary potential may decrease either because of ion carriers that were unaccounted for or may be a consequence of the core of the membrane being so highly selective for

anions that it reduces the effect of the negative charges at the surface. To decide between the two alternatives, it is necessary to determine t^+ as a function of surface charge density for a membrane wherein the core material is intrinsically non-selective. To do this, we make use of the general equation given by Scatchard (1955)

$$dE=\frac{RT}{F}[t^+ d(\ln c^+)-t^- d(\ln c^-)] \tag{14}$$

which describes the potential increment per concentration difference increment across a region characterized by transferrence numbers t^+ and t^-. Now, the diffusion potential which arises after shorting a bilayer membrane may be considered to be a liquid junction potential in which the ionic concentration gradients are taken to be those which appear across the membrane *per se*. Because of the surface charge, the surface concentrations will be considerably different from those of the bulk solution. It is this effect alone which we want to evaluate; so, to exclude considering the effect of the hydrocarbon, we take $t^+=t^-$. Eq. (14) then becomes

$$dE=\frac{RT}{2F}[d(\ln c_s^+)-d(\ln c_s^-)] \tag{15}$$

where c_s^+ and c_s^- are surface concentrations. Surface and bulk concentrations are related according to

$$c_s^+=c_b^+ e^{\frac{\varphi_0 F}{RT}} \tag{16}$$

$$c_s^-=c_b^- e^{-\frac{\varphi_0 F}{RT}}. \tag{17}$$

If the concentration on one side of the membrane is held constant and that on the other side is allowed to vary, the change of the surface concentration with respect to the bulk concentration of positive ions is given by the derivative of Eq. (16), i.e.,

$$dc_s^+=dc_b^+ e^{\frac{\varphi_0 F}{RT}}+\frac{F}{RT}c_b^+ e^{\frac{\varphi_0 F}{RT}} d\varphi_0, \tag{18}$$

or

$$dc_s^+=\frac{c_s^+}{c_b^+}dc_b^+ +\frac{\varphi_0 F}{RT}c_s^+ d\varphi_0. \tag{19}$$

Substituting for the value of $d\varphi_0$ from Eq. (2) and dividing by c_s^+ yields

$$\frac{dc_s^+}{c_s^+}=\frac{dc_b^+}{c_b^+}-\tanh\frac{F\varphi_0}{2RT}d(\ln c_b^+) \tag{20}$$

or

$$d(\ln c_s^+)=d(\ln c_b^+)\left(1-\tanh\frac{F\varphi_0}{2RT}\right). \tag{21}$$

Similarly for negative ions,

$$d(\ln c_s^-) = d(\ln c_b^-)\left(1+\tanh\frac{F\varphi_0}{2RT}\right). \tag{22}$$

Substituting Eqs. (21) and (22) into Eq. (15) and assuming the electroneutrality condition ($c_b^+ = c_b^- = c_b$), gives

$$\frac{dE}{d(\ln c_b)} = \frac{RT}{F}\tanh\frac{F\varphi_0}{2RT}. \tag{23}$$

Comparison of this equation with Eq. (7) shows that, when considered from the point of view of a liquid junction potential, a charged bilayer membrane is characterized by an effective positive ion transference number of

$$t^+ = \tfrac{1}{2}\left(1-\tanh\frac{F\varphi_0}{2RT}\right) \tag{24}$$

provided that the hydrocarbon interior is intrinsically non-selective. It is obvious from Eq. (24) that at zero surface charge $t^+ = 1/2$ and $t^+ \to 1$ at high negative surface charge density.

Our work, as well as the work cited in the Introduction, indicates that in bilayer membranes formed with hydrocarbon solvents, membrane concentration potentials are indeed very close to zero in uncharged membranes. But since the fatty acid compositions of the various phospholipids used in these experiments do not differ greatly, and there is no reason to suspect that one hydrocarbon environment would be more favorable for a particular charge than any other, we are forced to the conclusion that the surface charge of these membranes accounts entirely for the membrane potentials and that any decay to a value lower than that of the initial difference in double layer potentials must be caused by charge carriers which are available to be distributed uniformly across the membrane. Hopfer *et al.* (1970) have observed phenomena similar to those depicted in Fig. 10 and have suggested that significant proton conductivity may shunt the membrane potential. The membranes we have studied also exhibit appreciable potentials in the presence of pH gradients, sometimes as large as 58 mV per decade, and it appears likely that bilayers are sufficiently permeable to protons to render very tenuous any conclusions about the properties of the interior of membranes on the basis of concentration potentials. Dennis, Stead and Andreoli (1970) have observed very appreciable proton (or hydroxyl ion) conductivity in phospholipid-free bilayers; on the other hand, earlier work suggested that hydrogen plus hydroxyl ion conductances in membranes formed from red blood cell lipids is in the order of 10% or less (Andreoli, Tieffenberg & Tosteson, 1967).

It is of significance in this regard that the membranes which we studied have exhibited, at the very most, a very small dependence of conductance upon either charge density or salt concentration. Since conductance would be proportional to salt concentration and approximately proportional to exponential $F\varphi_0/RT$ if the ions of the added electrolyte were the primary current carriers, the relative constancy of the conductance in the two situations argues strongly for the presence of extraneous charge carriers. The lack of an effect of charge density is particularly incriminating, for there we would expect that our most highly charged membranes would have conductances $10^4\times$ greater than those of uncharged membranes. Such differences were indeed found by Eisenman, McLaughlin and Szabo (1970) in membranes doped with various ion carriers wherein any conductance from unknown ions must have been negligible. In our experiments, this difference could have been no larger than $4\times$. It should be stressed that this observation places rather severe limitations on the possibilities for the mechanism of conductance, for even if protons were the major current carriers, the conductance would still be surface charge dependent unless the rate-limiting step is unaffected by the concentration of protons in the membrane.

The authors wish to express their gratitude to N.G.A. Miller for assistance in the laboratory and in particular for the preparation of phosphatidic acid and phosphatidyl serine. R.C.M. is indebted to the Wellcome Trust for financial support.

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