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1-Anilino-8-Naphthalenesulfonate: A Fluorescent Indicator of Ion Binding and Electrostatic Potential on the Membrane Surface*

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Summary. The binding equilibrium of the hydrophobic fluorescence probe 1-anilino-8-naphthalenesulfonate (ANS^-) on lecithin (PC) membranes has been used to measure the electrostatic surface potential at the ANS^- binding site on the surface of these membranes. This method was used to study the variation of surface potential in lecithin membranes as a function of the ionic composition of the medium and as a function of the membrane surface charge. The latter was systematically varied by varying the degree of ANS^- binding and by incorporation of phosphatidic acid (PA).

Good agreement was found between the effects of ionic strength and surface charge on the surface potential calculated by the ANS^- binding method and the predictions of the Gouy-Chapman theory in an empirically modified form.

The ANS^- method was applied in conjunction with the modified Gouy-Chapman equations to the problem of cation binding to phospholipid membranes. The analysis showed that none of the monovalent (M^+) and divalent (M^{2+}) cations studied here bind to the polar head groups of PC. Binding of M^{2+} to PA^- (and PA^{2-}) incorporated in PC membranes was demonstrated. The apparent binding constant for this reaction is given as $K_2 = K_{20} * (f(\sigma)^{-2})$ where K_{20} is the "chemical" binding constant which is independent of the surface potential and where $f(\sigma)$ is an exponential function of membrane surface potential. The validity of this analysis regarding M^{2+} binding and the separation of surface potential-dependent and independent contributions was supported by parallel experiments using murexide as an indicator of Ca^{2+} in the aqueous phase.

The value of K_{20} for Ca^{2+} is about 200 M^{-1}. This value is within an order of magnitude of the corresponding binding constant for Ca^{2+} and $H_2PO_4^-$ in solution, indicating that the two processes are quite similar. Both the value of K_{20} and its ion specificity ($La^{3+} \gg Mn^{2+} \sim Mg^{2+} > Ca^{2+} > Ba^{2+}$) are essentially independent of the mole fraction of PA in the PC membrane. It is shown that M^{2+} binding can influence the distribution of PA^- within the PC membrane, especially for low mole fractions of PA^-.

The modification of the Gouy-Chapman equations used in the present study is discussed in terms of discreteness of charge effects, and it is concluded that caution must be exercised in applying the theory in unmodified form to the binding reactions of

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ions to specific charged or uncharged sites on the membrane surface. The applicability of the ANS^- method to the determination of cation binding and electrostatic potential on the surfaces of more complicated biological membranes is discussed.

Membrane electrostatic surface potential is an important functional property of biological membranes. It determines the partition of ions between the aqueous phase and the membrane surface and thus affects their binding, and it undoubtably plays an important role in processes such as membrane aggregation and fusion. The determination of its value is a difficult problem which is not easily attacked by experimental means in complicated membrane systems.

The present communication expands on my preliminary report [13] that the binding of the hydrophobic fluorescent probe 1-anilino-8-naphthalenesulfonate (ANS^-) to phospholipid membranes responds to the membrane surface potential, and shows how the probe can be used as a general indicator of electrical potential near the membrane surface. Similar methods involving the effect of membrane electrostatic interactions with a membrane-bound pH indicator [16] and on the ionophore-mediated K^+ transport in lipid bilayer membranes [12, 15] have been reported. These methods are compared with the ANS^- method in the present communication.

A knowledge of the membrane surface potential allows the calculation of the net membrane charge and the measurement of ion binding reactions, provided that the relationship between surface potential, surface charge and electrolyte composition and concentration is known. The Gouy-Chapman theory (*cf.* [17]) gives a mathematical relationship between these parameters and predicts the course of potential drop from the membrane to the aqueous solution (i.e., the distribution of counter-ions in the electric double layer). The application of this theory, or a similar poly-electrolyte theory, is necessary to distinguish between the effects of ionic composition of the medium and ion binding on the above-mentioned reactions. Previous studies on cation binding effects in phospholipid vesicles [1, 18] and in sarcoplasmic reticulum [9, 23] have not distinguished between these two effects whose importance has recently been emphasized [15]. In the present study, this type of analysis is applied to the problem of divalent cation binding to phosphatidic acid (PA) incorporated within lecithin (PC) membranes, using the ANS^- as an indicator of electrostatic potential at the membrane surface.

Materials and Methods

The sources and quality of the reagents and lipids have been described in the previous paper [14]. The vesicle preparations were treated with 10^{-5} M EDTA and then

dialyzed overnight as a precaution against effects of heavy metals which might be introduced during the sonication. All experiments were carried out at lipid concentration 6.8×10^{-4} M unless otherwise indicated.

The degrees of ANS$^-$ binding were calculated as described in the companion paper [14] using the appropriate values of Q from Table 3. Two types of experiment have been carried out in the present study: In type (a) the electrolyte composition was held constant and the ANS$^-$ concentration is varied, while in type (b) the ANS$^-$ concentration is held low and constant and the electrolyte composition is systematically varied. In experiments of type (a) the ANS$^-$ binding reaction can make a substantial contribution to the total membrane surface charge whereas in experiments of type (b), the ANS$^-$ binding responds to pre-existing charges. Thus, type (a) experiments can give the value of K_{10} and $[B]_t$ and the variation of $\exp(e\psi_0/kT)$ ($f(\sigma)$ here) with membrane surface charge and ionic composition. Experiments of type (b) were used to calculate K_{10} and the divalent cation binding constant for the membrane, K_{20}.

All experiments consisted of at least 10 experimental points and all calculations were made on a Univac 1108 computer. Values of ANS$^-$ binding were calculated by a subroutine which can be described by the following steps: (a) Calculation of binding of all species using their aqueous concentrations, equilibrium constants given and the $f(\sigma)$ value of the $(n-1)$th calculation. (b) Calculation of the surface charge, σ, as the algebraic sum of the charges of these membrane-bound species. (c) Calculation of ρ_{eff} from the given electrolyte concentrations and the $(n-1)$th value of $f(\sigma)$. (d) Calculation of σ_{eff} from ρ_{eff} and σ using the dependence assumed. (e) Calculation of $f(\sigma)$ from σ_{eff} and the given electrolyte composition according to the Gouy-Chapman equations. (f) Returning to step (a). This procedure was repeated for each calculation until the calculated bound concentrations of all species converged within 0.0001. For systems with ions of higher valency than 1, step (e) required that the Gouy-Chapman equations be solved by the method of Newtonian approximation. When values of $f(\sigma)$ were described according to the unmodified Gouy-Chapman equations, σ_{eff} was put equal to σ.

The binding constants for the ionic species were determined by fitting these parameters in the main program by a procedure of least-squares minimization of the difference between the experimental and the calculated data relative to the experimental data. In experiments of type (a), K_{10} and a second fitted parameter ($[B]_t$ or k_ρ) were allowed to vary independently. When no clear least-square minimum was found, one parameter was fitted at a time and this procedure was repeated until the best result was obtained. In experiments of type (b), the value of K_{10} was first calculated for zero divalent cation concentration and this value was held constant while the value of K_{20} or other parameters were fitted for the rest of the data.

The goodness of fit was determined as the relative standard deviation of the mean, SD, based on the difference between the experimental and calculated values divided by the experimental value. The value of this parameter had to be less than 0.1 and there could be no evidence for systematic deviations for the fitted parameters to be considered usable. The uniqueness of the fitted parameters was evaluated by the effect of their variation $\pm 30\%$ on SD.

Both the experimental and calculated data were plotted by a subroutine, and the solid lines of the figures in this study represent the calculated data for the fitted constants.

Murexide Experiments

Ca^{2+} binding to vesicles containing PA$^-$ was determined from Ca^{2+} titrations based on difference spectra taken on the Cary 14 spectrophotometer, using murexide as an absorption indicator for Ca^{2+} in the aqueous phase. Buffered solutions (19 mM

Tris Cl) of 8.6×10^{-5} M murexide always were used on both cuvettes. Difference spectra were taken for the configuration (murexide; buffer; *variable* [Ca^{2+}]) *vs.* (murexide; buffer) and the difference in absorption at 550 nm was analyzed to determine the binding constant of Ca^{2+} for the indicator, K_s, and the maximal change in absorption upon saturation with Ca^{2+}, ΔAbs_{max}. The Ca^{2+} binding to vesicles was studied by taking difference spectra for the configuration (murexide; buffer; variable [Ca^{2+}]; *vesicles*) *vs.* (murexide; buffer; variable [Ca^{2+}]). The change in absorption ΔAbs was meaured and corrected for the effect of Ca^{2+} addition on the absorption signal arising from light scattering of the vesicles. This effect, which increases with decreasing wavelength, could be adequately evaluated by linear interpolation of the baseline of the absorption signal between 650 and 510 nm, the isobestic point for the murexide difference spectrum. It was also necessary to correct ΔAbs for a small (ca. 2%) unmatchedness of the cuvettes according to:

$$\Delta Abs = \Delta Abs_{measured} - \Delta Abs_{\infty} * S \tag{1}$$

where ΔAbs_{∞} is the maximum difference in absorbance between identically filled cuvettes containing a saturating concentration of Ca^{2+} and where S is equal to the fraction of the indicator complexed with Ca^{2+}:

$$S = [Ca^{2+}] * K_s/(1 + [Ca^{2+}] * K_s). \tag{2}$$

All binding constants in the system are low enough that the free concentration of Ca^{2+} is nearly identical to the total concentration. The concentration of Ca^{2+} bound to the phospholipid, $[Ca^{2+}]_b$, is thus given as:

$$[Ca^{2+}]_b = [Ca^{2+}] - \frac{A}{K_s * (1 - A)} \tag{3}$$

where

$$A = S - \frac{\Delta Abs}{\Delta Abs_{max}}. \tag{4}$$

Results and Discussion

Electrostatic Influence on ANS^- Binding

Fig. 1 shows the effect of ionic strength on ANS^- binding. At high electrolyte concentrations a linear double reciprocal plot is obtained, in agreement with the binding site model of Eq. (4) of the companion paper [14] and indicating that the value of K_1 is constant. Decreasing the ionic strength shifts the curves in the direction of decreasing binding and results in a curvature of the double reciprocal plot. This indicates that at low ionic strength, the ANS^- binding becomes anticooperative in nature, with increasing degrees of binding serving to decrease the binding affinity.

Attempts to fit the data of Fig. 1 to models involving cation binding to the ANS^- on the membrane surface as described by other workers (*cf.* [13]) were unsuccessful. It must thus be concluded that this inhibition of ANS^- binding is due to the effect of the negative surface charge brought about by the binding of the anionic probe itself. I have previously formulated the

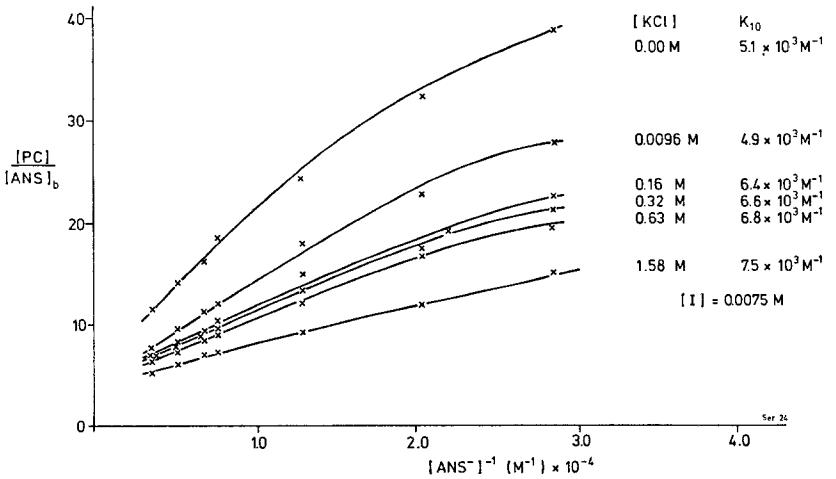

Fig. 1. Double reciprocal plot of ANS^- binding to dimyristoyl PC monolayer vesicles at 30 °C. The pH was held constant at 7.3 with 7.5 mM imidazole buffer (*I*), and the variable KCl concentrations are indicated in the figure. The solid lines are the result of the computer analysis, arriving at $[B]_t = 0.25$, $k_\rho = 103\ M^{-1}$ and the indicated K_{10} values. All lines converge for degrees of binding lower than shown here

dependence of the binding constant K_1 (*cf.* Eq. (4), [14]) on the membrane surface potential ψ_0 according to [13]:

$$K_1 = K_{10} * \exp(e\psi_0/kT) \tag{5}$$

where K_{10} is the binding constant at high ionic strengths and low membrane surface charges, where k is the Boltzmann constant and T is the absolute temperature. Thus, the value of the membrane surface potential can be calculated from the experimentally determined values of K_1 and K_{10} if it is assumed that the experimental ionic strength perturbations have no influence on K_{10}, the "chemical" binding constant, and the ANS^- does not ion pair with cations in the medium or associate with protons.

I have reported [13] that the ANS^- binding behavior can be calculated using the dependence of ψ_0 on membrane surface charge, σ, and electrolyte valency and concentration given by the Gouy-Chapman equation (*cf.* [17]). It is possible to apply this theory for a planar membrane to the case of the curved monolayer vesicle since the radius of curvature is much larger than the Debye-Hückel distance $1/\kappa$ (*cf.* [17]). In its most general form, the equation can be written:

$$\frac{\sigma^2}{\frac{\varepsilon * kT}{2\pi}} = \Sigma n_{i0} * [\exp(-z_i e\psi_0/kT) - 1] \tag{6}$$

where z_i is the charge of the ionic species with concentration n_{i0} in the bulk solution, where ε is the dielectric constant of the solution and where the summation is carried over all species in the solution. In the case of a 1:1 electrolyte, Eq. (6) reduces to:

$$\exp(\pm e\psi_0/kT)=\frac{\left(\frac{\sigma^2}{\frac{\varepsilon*[M^+]*kT}{2\pi}}+2\right)+\left(\left(\frac{\sigma^2}{\frac{\varepsilon*[M^+]*kT}{2\pi}}+2\right)^2-4\right)^{\frac{1}{2}}}{2} \tag{7}$$

where the choice of $\pm$ depends upon the sign of σ, and where $[M^+]$ is the univalent electrolyte concentration. For the case of mixed 1:1 and 2:1 electrolytes, the dependence is more complicated:

$$\frac{\sigma^2}{\frac{\varepsilon*kT}{2\pi}}=[M^+](\exp(e\psi_0/kT)+\exp(-e\psi_0/kT)-2) +[M^{2+}](2\exp(e\psi_0/kT)+\exp(-2e\psi_0/kT)-3). \tag{8}$$

The value of $f(\sigma)=\exp(e\psi_0/kT)$ can be solved from the resulting trinomial. In a similar manner, the relationships for 3:1 electrolytes such as $LaCl_3$ have been derived and used in this study.

Eq. (7) can be used to fit the experimental data in the membrane system of Fig. 1 when the calculated value of $f(\sigma)$ is low. The degree of ANS^- binding is calculated using a value of $[B]_t$ determined at high ionic strength and using values of K_1 calculated from $f(\sigma)$ and the fitted value of K_{10}. In these calculations the dielectric constant of bulk water and a molecular area of 58 $Å^2$ per lipid molecule [3, 22] were used. For the experimental data corresponding to $f(\sigma)$ values, good agreement between the observed and calculated binding data was found using $[B]_t=0.25$ and a constant value of K_{10}. However, under the conditions of high degrees of binding and low ionic strength it was not possible to fit the data with a constant value of K_{10}.

The deviations between the calculated and experimental data were much larger than could be explained by variation of the surface area per lecithin molecule or by variation of the dielectric constant of water near the membrane surface. Several other assumptions were applied unsuccessfully in an attempt to apply Eq. (7) to the data of the left-hand portion of Fig. 1. It could not be assumed that ANS^- bound in the protonated form since the data of Fig. 1 are independent of pH between values of 3 and 10. The assumption that the ANS^- contribution to σ is diminished by M^+ binding

to the ANS$^-$ in response to a chemical binding constant and the calculated membrane surface potential had several drawbacks: (a) The data are virtually independent of whether M^+ is Na^+, K^+, Rb^+ or Cs^+. (b) The assumption of cation binding constants large enough to obtain a fit of the data with constant K_{10} and $[B]_t$ values made the ANS$^-$ binding totally insensitive to the electrostatic effects of M^{2+} addition in the lecithin and lecithin-PA systems of this study. (c) A similar discrepancy in the region of $f(\sigma) \ll 1$ is found in experiments similar to that of Fig. 1 in which K^+ is replaced by M^{2+}. No M^{2+} specificity is found in these experiments, and ion pairing of Mn^{2+} with ANS$^-$ is ruled out in these experiments by the lack of observation of fluorescent quenching [14].

It was next assumed that the potentials calculated by the Gouy-Chapman equations were too high because the charges on the membrane surface are partially screened by the counter-ions in a way not predicted by the Gouy-Chapman theory. The surface potential would thus be calculated by the Gouy-Chapman equations using a $\sigma_{\text{eff}} (< \sigma)$ value which would represent the effective surface charge seen by the ions in the double layer. The problem was thus to find how σ_{eff} depends upon σ and ionic strength. The quotient $\sigma_{\text{eff}}/\sigma$ was analyzed in several experiments and plotted as a function of ionic strength and σ and was found not to have a simple dependence on either of these parameters. However, it was found to decrease with increasing space charge in the aqueous phase at the membrane surface, $\rho_{\text{eff}} = \Sigma z_i n_i$ where n_i is the concentration of the ith species at the membrane surface. ρ_{eff} was calculated for the known ionic composition of the solution and σ_{eff}.

The value of σ_{eff} seemed to deviate from σ according to:

$$\sigma_{\text{eff}} = \sigma/(1 + k_\rho * \rho_{\text{eff}}) \tag{9}$$

where k_ρ is a constant proportionality.

Use of Eq. (1) in conjunction with Eq. (8) in the iterative computer procedure described in Materials and Methods resulted in a good fit between the experimental data and the calculated values, as shown in Fig. 1. The constant $k_\rho = 103\ \text{M}^{-1}$ gave a good fit of the data, with the value of the fitted constant K_{10} showing only a small variation over a 100-fold variation of the ionic strength of the medium. The values of k_ρ and K_{10} found here were successful in predicting the degrees of ANS$^-$ binding found in similar experiments of type (a) where the concentration of M^{2+} was systematically varied. We have thus arrived at an empirical equation and constant which modify the Gouy-Chapman equations to give agreement between the theory and results. It is noted that under the experimental conditions given here

the use of $k_\rho = 103\ \text{M}^{-1}$ has the effect of reducing σ by a constant amount. The physical meaning of the correction will be discussed in another section.

Cation Binding

This section shows how the effect of cation addition to increase ANS^- binding can be used to determine the degree of cation binding to negatively-charged phospholipids, making use of the effect of cation binding to decrease the negative membrane surface potential. This is accomplished in experiments of type (b) where the degree of ANS^- binding is first determined for fixed concentration of ANS, vesicles and KCl, in the absence of divalent cation. The suspension is then titrated with MCl_2 and the increase in ANS^- binding is recorded. Evaluation of the binding constants is done in a completely analogous fashion: The value of K_{10} is calculated for the condition $[M^{2+}]=0$, and the constant and the data for finite M^{2+} concentrations are used to fit the value of K_{20}, the cation binding constant for the negative site.

Fig. 2 is the result of the above analysis for the interaction of Ca^{2+} with dimyristoyl PC monolayer vesicles. Increases in $[Ca^{2+}]$ result in increased shielding of the negative charge imparted to the membrane by the bound ANS^-, making further binding possible. The effect increases with increasing $[ANS^-]$. Repetition of the experiment of Fig. 2 for successively higher 1:1 electrolyte concentrations results in a family of similarly shaped curves shifted upwards and converging at high $[M^{2+}]$. This con-

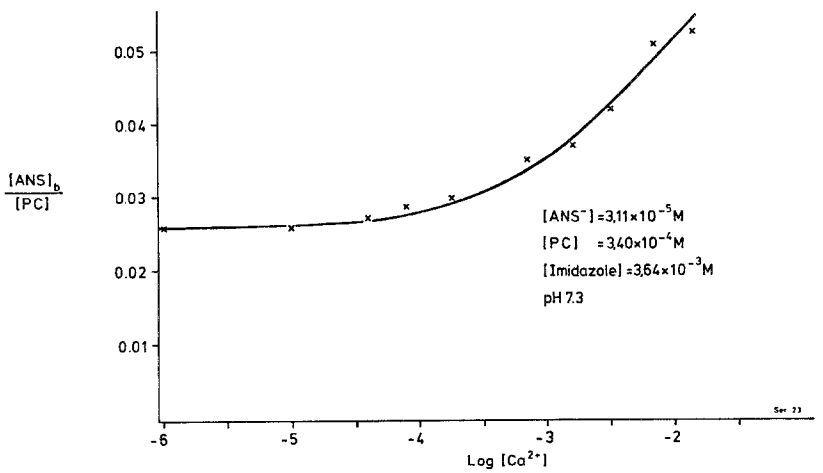

Fig. 2. The effect of Ca^{2+} addition on ANS^- binding to dimyristoyl PC monolayer vesicles at 30 °C. The experimental conditions are given in the figure. The solid line represents the computer fit of the data using $[B]_t = 0.25$ and $k_\rho = 103\ \text{M}^{-1}$

vergence indicates that the Ca^{2+} effect is almost completely due to the influence of Ca^{2+} in the double layer on the ANS$^-$-induced surface charge. Direct binding of Ca^{2+} on lecithin sites or on the ANS$^-$ to produce a net positive charge on the membrane would result in nonconvergence of the curves at high $[M^{2+}]$.

This conclusion is supported by the results of the analysis of these data using Eqs. (4) and (5) with $k_\rho = 103\ M^{-1}$ calculated with the help of the computer program. A binding constant for ANS of $K_{10} = 1.03 \times 10^4$ was determined. This value was independent of $[M^+]$ for $k_\rho = 103\ M^{-1}$, but was found to vary when a substantially larger or smaller value of k_ρ was used in the analysis. Analysis of the data for finite Ca^{2+} concentration under the assumption that Ca^{2+} could bind to the polar head groups of lecithin gave a nonelectrostatic binding constant (analogous to K_{10} in Eq. (1) [14]) of $0.7\ M^{-1}$ for this reaction. Since the fit of the data was practically as good when it was assumed that this binding could not take place, it is concluded that the binding is practically nil and that this value should be considered as an upper limit. Supporting this conclusion is our observation that Mn^{2+} cannot penetrate the polar head group region of lecithin membranes [14] and that the effect of Fig. 2 is completely devoid of divalent cation specificity.

Repetition of the experiment of Fig. 2 using La^{3+} instead of Ca^{2+} gave evidence for La^{3+} binding to the lecithin membrane with a nonelectrostatic binding constant for the polar head groups in the range 250 to $1{,}000\ M^{-1}$.

The effects of M^{2+} addition on ANS$^-$ binding are much larger when the lecithin membranes contain PA as a negative binding site for the added cations. Figs. 3 and 4 show the effect of ionic strength and Ca^{2-} concentration on ANS$^-$ binding to dimyristoyl PC/PA monolayer vesicles with mole ratios 9:1 and 7:3, respectively, at pH 7.3 where PA carries a single negative charge. The lower right portions of the figures show the effect of increasing the monovalent cation concentration to 1.5 M. The degree of binding is increased to a maximum value. The effect of Ca^{2+} addition is to increase the degree of ANS$^-$ binding above this value. Anticipating the discussion to follow, the effect of the added Ca^{2+} is to increase the electrostatic shielding of the surface charge, and to make the surface charge more positive by the mechanism of direct binding of the PA. The direct binding also affects the distribution of PA within the lecithin matrix, thus increasing the number of ANS$^-$ binding sites. The discussion below will describe the model used to quantitate the effect of M^{2+} on ANS$^-$ binding. This will be followed by a description of unsuccessful attempts to fit the data with

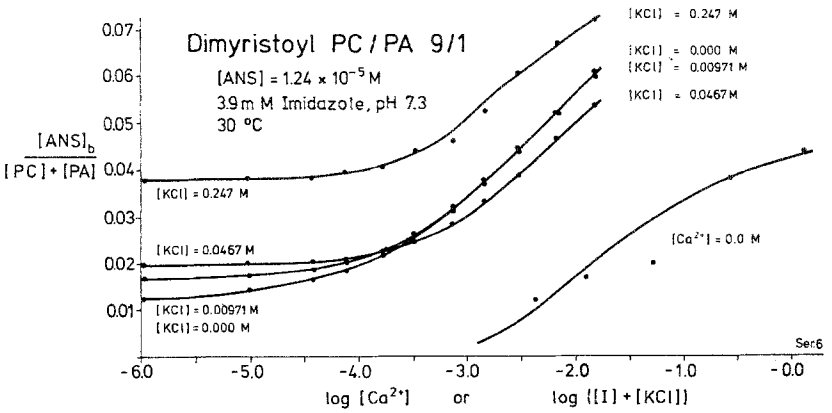

Fig. 3. The effect of Ca^{2+} on ANS^- binding to dimyristoyl PC/PA monolayer vesicles — mole ratio 9:1. The experimental conditions are given in the figure. The solid lines represent computer fits of the data, using $k_\rho = 103\ M^{-1}$ and Eqs. (12) and (13). The values of the fitted constants K_{10} and K_3 are given in Table 1

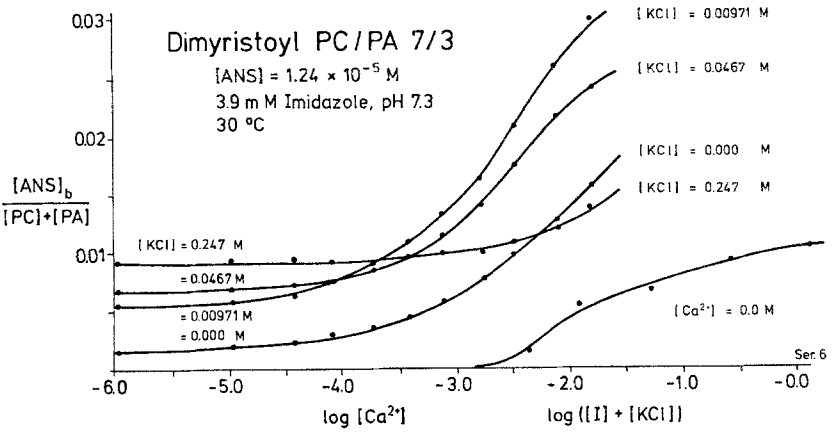

Fig. 4. The effect of Ca^{2+} on ANS^- binding to dimyristoyl PC/PA monolayer vesicles; mole ratio 7:3

other models. Finally, an independent check for the correctness of this model is given.

The modification of the Gouy-Chapman equations given in Eq. (9) was assumed to be necessary with $k_\rho = 103\ M^{-1}$. Analysis of the data for variation of the M^+ concentration in the absence of M^{2+} was analyzed according to these assumptions, using -1 as the charge on the PA, 58 $Å^2$ as the area per lipid molecule, and using the value of $[B]_t$ from Eq. (10) of the companion paper [14]. The values of K_{10} determined by this procedure are essentially independent of the PA content and the M^+ concentration,

indicating that there is little tendency of M^+ to bind on the PA. The solid lines going through the data points of Figs. 3 and 4 were generated by the computer program, using the average values of K_{10}.

The data for variable Ca^{2+} concentration was analyzed, using the fixed values of K_{10} determined above. It was assumed that Ca^{2+} can bind to the PA according to the reaction:

$$Ca^{2+}_{aq} + PA^-_{memb} \overset{K_2}{\leftrightarrow} (Ca-PA)^+_{memb} \tag{10}$$

and that the net surface charge of the membrane is modified to the extent that this binding takes place. The Ca^{2+} binding equilibrium was assumed to respond to the membrane surface potential in the same way as does ANS$^-$, with the bound Ca^{2+} concentration, $[Ca^{2+}]_b$ given by:

$$[Ca^{2+}]_b = \frac{[PA]_t * K_{20} * [Ca^{2+}] * (f(\sigma)^{-2})}{1 + K_{20} * [Ca^{2+}] * (f(\sigma)^{-2})} \tag{11}$$

where K_{20} is the "chemical" binding constant for Ca^{2+} directly analogous to K_{10} for ANS$^-$. It was necessary to assume that the Ca^{2+} binding could influence the distribution of PA within the membrane. An association between PA and Ca–PA was assumed to take place, and this association was assumed to remove one PA from a four-membered lecithin binding site, such that:

$$[B]_t = [B]_a * (1 - [PA]_t + K_3 * [Ca^{2+}]_b * [PA]/[PA]_t)^4 \tag{12}$$

where $[B]_a$ is the number of binding sites per lecithin molecule for lecithin membranes (0.25), where $[PA]_t$ represents the total concentration of PA in the system, and where all concentrations are normalized to the total lipid concentration (*cf.* Eq. (10), Ref. [14]). K_3 can be understood as a sort of bimolecular association constant for Ca^{2+}-PA and PA on the membrane surface. The model thus predicts that Ca^{2+} binding will initially increase the number of binding places, but when a stoichiometry of 0.5 Ca^{2+}/PA has been reached, further Ca^{2+} will result in a decrease in the number of ANS$^-$ binding sites.

The data of Figs. 3 and 4 were fitted using Eqs. (9), (11) and (12), and the values of K_3 and K_{20} determined by this analysis are given in Table 1. The values of K_{20} vary only a factor of 7 for a fivefold variation of surface charge and a 100-fold variation of ionic strength. Against the background of the 10^4-fold variation predicted by the Gouy-Chapman theory for K_2 over this range of experimental conditions, K_{20} shows negligible variation.

In the course of this work, numerous alternative explanations for the effect of Ca^{2+} on ANS$^-$ binding were attempted. Models involving direct

Table 1. Constancy of K_{10} and K_{20} with PA content and ionic strength

Mole fraction PA	KCl (M)	K_{10} ($M^{-1} \times 10^{-4}$)	K_3	K_{20} (M^{-1})	SD
0.1	0.000	2.93±0.90 (3.50)	0.44±0.20 (0.096)	176±30 (149)	0.05
0.1	0.00971	1.94±0.45 (2.54)	3.80±0.20 (4.0)	255±60 (368)	0.05
0.1	0.0467	1.59±0.40 (1.67)	3.80±0.20 (4.0)	311±45 (352)	0.05
0.1	0.249	1.78±0.30 (2.79)	2.67±0.50 (4.0)	120±40 (300)	0.05
0.2	0.000	3.60±1.40	0.40±0.15	100±50	0.11
0.2	0.00971	1.55±0.40	0.80±0.30	292±30	0.055
0.2	0.0467	1.10±0.30	0.44±0.10	175±40	0.08
0.2	0.249	1.24±0.30	1.00±0.30	280±50	0.09
0.3	0.000	1.30±0.07 (2.40)	0.00 (0.00)	100±30 (94)	0.08
0.3	0.00971	2.80±0.60 (2.17)	0.92±0.20 (0.92)	249±30 (268)	0.08
0.3	0.0467	1.40±0.30 (1.88)	1.32±0.32 (1.51)	355±70 (442)	0.05
0.3	0.249	1.43±0.30 (1.72)	0.92±0.08 (0.40)	240±60 (187)	0.05
0.5	0.000	10±4	0.77±0.20	50±20	0.10
0.5	0.00971	6±2	0.55±0.20	167±30	0.10
0.5	0.0467	3.2±1.0	0.60±0.20	221±50	0.08
0.5	0.249	4.7±2.0	0.54±0.20	170±30	0.06

Experimental conditions: Dimyristoyl PC/PA monolayer vesicles, 3.9 mM imidazole buffer, pH 7.3, 1.24×10^{-3} M ANS^-, 30 °C. All measurements were repeated at least 6 times on three separate preparations, and the ± values give the standard deviation of the mean. The values in parentheses are those determined in Figs. 3 and 4.

binding with no electrostatic interaction were completely unsuccessful. Attempts at using the Gouy-Chapman equations in unmodified form required the assumption of M^+ binding on the membrane-bound ANS^- or on the PA. The degree of M^+ binding on the ANS^- necessary to achieve constancy in K_{10} predicted that the ANS binding would be practically unresponsive to the effects of divalent cations both in the absence and in the presence of PA. The assumption of M^+ binding on the PA required "chemical" binding constants of the order of 60 M^{-1} to achieve constancy in K_{10}. The Ca^{2+} binding equilibrium was then in direct competition with the M^+

binding equilibrium. This assumption had to be rejected because it resulted in Ca^{2+} binding constants, K_{20}, which increase with increasing M^+ concentration, showing a four order of magnitude variation for $0.0039 < [M^+] < 0.247$ M.

Experiments with Murexide

The preceding analysis would seem to indicate that the ANS$^-$ binding reaction is a good indicator for the binding of cations on the membrane surface. However, the necessity of assuming the variation of $[B]_t$ with Ca^{2+} binding according to Eq. (12) has introduced an additional adjustable parameter, K_3, thus increasing the complexity of the analysis. To obtain an independent check of the conclusions here, experiments of the type shown in Figs. 3 and 4 were performed in parallel with experiments measuring the actual Ca^{2+} binding. This was accomplished using murexide as an indicator for the Ca^{2+} concentration in the aqueous phase [20].

At the pH value of 8.5, the condition under which the experiments reported here were carried out, the net complexation reaction of Ca^{2+} with the indicator is described as:

$$H_4F^- + Ca^{2+} \overset{K_s}{\leftrightarrow} H_3F - Ca + H^+ \tag{13}$$

where F denotes the indicator [20]. Since the complex is uncharged, the indicator is not influenced by the electric potential about the membrane surface, and thus serves as a true indicator of the total Ca^{2+} which is not bound to the membrane surface. Since the murexide technique offers a direct experimental means of registering direct binding, it is a much better method for distinguishing binding from double layer effects than the indirect surface potential calculations given here and elsewhere [15].[1]

A preparation of dimyristoyl PC/PA (5:5) monolayer vesicles was subjected to the procedure and analysis of Fig. 3 in the presence and absence of 8.6×10^{-5} M murexide at pH 8.5. Potentiometric titrations indicated that under these conditions the PA carried an average charge of -1.3. A Ca^{2+} binding constant $K_{20} = 190 \pm 10\ M^{-1}$ was obtained using the titration procedure of Fig. 2 both in the absence and presence of 0.3 M NaCl with 19 mM Tris-Cl. The same constant was obtained both in the absence and

1 Even below pH 8.2 where the product of reaction given in Eq. (13) is $H_4F - Ca^+$, it can be shown by numerical integration of the Gouy-Chapman equation for potential distribution in the double layer that the effect of electrostatic potential on the performance of murexide as an indicator would be negligible for the conditions of the present study. In any case, the effect would cause the degree of Ca^{2+} binding to the membrane to be *underestimated.*

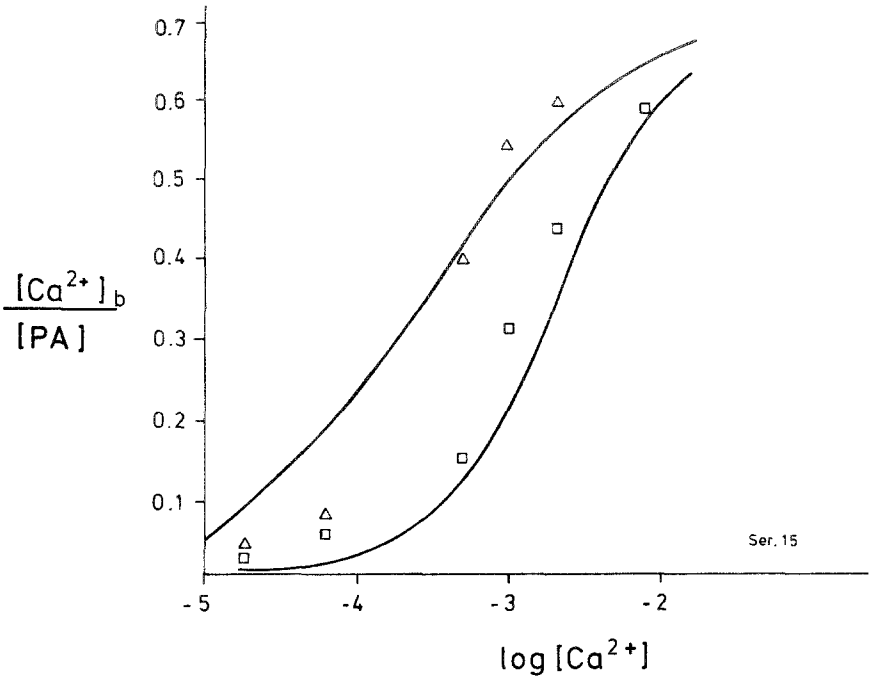

Fig. 5. Comparison of the degrees of Ca^{2+} binding calculated by the ANS^- and murexide methods. The experimental conditions are described in the text. The points represent values obtained by the murexide technique (△, [NaCl]=0.0 M; □, [NaCl]=0.3 M) and the curves represent the calculations from the fitted ANS^- binding data. The stability constants for the Ca^{2+}-murexide complex were 1,316 and 916 M^{-1} for 0.0 and 0.3 M NaCl, respectively

presence of murexide, indicating that the murexide does not bind or influence the membrane surface potential at these concentrations. Similarly, the murexide had no influence on the binding or the fluorescence signal of ANS^-, other than a small expected inner filter effect of the ANS^- fluorescence emission (*cf.* [21]). Since there is substantial overlap between the emission spectrum of ANS^- and the absorption spectrum of murexide, binding of the latter to the membrane, had it occurred to any appreciable extent, would have resulted in quenching of ANS^- fluorescence by the mechanism of quantum transfer [8].

Ca^{2+} binding experiments with murexide were performed with identical preparations and concentrations, using the analysis described in Materials and Methods. Fig. 5 compares the degrees of Ca^{2+} binding determined for this preparation by the two methods. The solid lines represent the degrees of binding determined by the computer analysis of the ANS^- experiments and the points are the values determined from the murexide experiments.

Table 2. The influence of ionic strength on the apparent binding constant for Ca^{2+} binding at pH 7.3

Mole fraction PA	1:1 Electrolyte (M)	K_{20} (M^{-1})	$K_{app\,(1/4)}$ (M^{-1})
0.1	0.0039	176	5.9×10^3
0.1	0.0136	255	2.4×10^3
0.1	0.0506	311	1.6×10^3
0.1	0.2529	120	4.4×10^2
0.2	0.0039	100	8.3×10^3
0.2	0.0136	292	5.3×10^3
0.2	0.0506	175	1.1×10^3
0.2	0.2529	280	1.0×10^3
0.3	0.0039	100	1.6×10^4
0.3	0.0136	249	6.7×10^3
0.3	0.0506	355	2.8×10^3
0.3	0.2529	240	1.0×10^3
0.5	0.0039	50	1.4×10^4
0.5	0.0136	167	7.7×10^3
0.5	0.0506	221	2.2×10^3
0.5	0.2529	170	7.7×10^2
1.0	0.019	$(\sim 280)^a$	$2.8 \pm 0.4 \times 10^{3\,b}$
1.0	0.319	$(\sim 500)^a$	$1.6 \pm 0.4 \times 10^{3\,b}$

[a] Estimated from the value of $K_{app\,(1/4)}$ using the relationship between $f(\sigma)$ and σ given in Fig. 6. A full analysis according to the electrostatic model given in the text was not attempted because the murexide technique was useful only for a limited range of Ca^{2+} concentration.

[b] Determined at pH 8.50 using the murexide technique, with 19 mM Tris buffer and 0 or 0.3 M NaCl.

The value of $K_{app\,(1/4)}$ is defined as the Ca^{2+} necessary for 1/4 saturation of the PA binding sites and half-neutralization of the membrane surface charge. Its value was calculated from the corresponding value of K_{20}, using a computer program to solve Eqs. (9), (11) and (12). The values are valid for the conditions of Table 1.

The latter method was rather inaccurate at low and high degrees of complexation where only small values of ΔAbs [*cf.* Materials and Methods, Eq. (3)] were observed and where the subtraction of the baseline becomes a problem. The otherwise good agreement between the two methods suggests that the ANS$^-$ method as used here is competent to measure the degrees of divalent cation binding.

Binding of Ca^{2+} to vesicles composed solely of PA could be measured by the murexide technique between pH 8.0 and pH 9.5 and values of the apparent binding constant for pH 8.5 are included in Table 2. Although the interpretation of the murexide experiments is much simpler than with the

ANS^- method, the former method has the disadvantage that the absorption changes are small and reliable data are obtained over a fairly narrow range of concentration. For this reason, the data were not subjected to an extended analysis using Eqs. (8), (9) and (11).

Dependence of Ca^{2+} Binding

With the murexide experiments confirming the ANS^- method of analysis of M^{2+} binding, we are now justified in considering the results of Table 1 in more detail. The value of K_3 gives a measure of the tendency for Ca^{2+} binding to PA^- to remove a second PA^- molecule from a four-membered ANS^- binding site. For low mole fractions of PA this can be accomplished by association of a PA^- with the $Ca^{2+}-PA^-$ complex to form a 2:1 complex or simply by electrostatic attraction of the PA^- by the $Ca^{2+}-PA^-$ complex such that the distribution of the former in the membrane is nonrandom. For larger mole fractions of PA, this complexation or association will not necessarily result in the liberation of an ANS^- binding site, and large values of K_3 approaching the theoretical maximum of 4.0 will indicate association and the formation of a separate $PA^- - Ca^{2+} - PA^-$ phase.

For the mole fraction 0.1 PA, the value of K_3 approaches 4.0 indicating that Ca^{2+} bound as $Ca^{2+} - PA^-$ can attract a second PA molecule. However, this need not be taken as evidence that a 2:1 complex is obligatory, and the observed constancy of K_{20} with mole fraction PA speaks against this. At higher mole fractions, lower values of K_{20} are determined, indicating that there is little tendency of the Ca^{2+} to cause formation of a separate PA phase. Ca^{2+} binding thus serves to neutralize the charge on the PA^- molecule allowing a second molecule to approach the complexed molecule more closely. Such effects of redistribution of molecules and charge on the membrane surface may play an important role in the effects of Ca^{2+} and Mg^{2+} in lipid-protein interaction, membrane aggregation and excitation in nerve.

Papahadjopoulos [18, 19] has proposed a model for Ca^{2+} binding on PA membranes, in which Ca^{2+} interacts with four PA molecules. Although no evidence for this could be found in the present study, it is possible that such a structure may contribute to Ca^{2+} binding in membranes composed of pure PA.

Table 2 compares the values of K_{20} with the apparent binding constants based on the concentration of Ca^{2+} necessary to 1/4 saturate the PA^- and half neutralize the membrane surface charge. The apparent binding constant for full neutralization of the membrane surface charge and for half saturation of the PA^- is identical to K_{20}, of course. The apparent binding

constant for half neutralization decreases with increasing ionic strength, and increases with increasing PA content, as is expected from the dependence of the Ca^{2+} binding on membrane surface potential. An important prediction arising from the present analysis is that at high Ca^{2+} concentrations, the membranes will gain a net positive charge.

Abramson and co-workers [1] have reported binding constants for Ca^{2+} and Mg^{2+} on PA membranes. M^{2+} binding was measured from the number of equivalents of tetramethylammonium hydroxide (TMAOH) necessary to maintain constant pH in these dispersions upon addition of the cation. In this study it was assumed (a) that the divalent cation reacted only with the doubly charged form of PA, and (b) that the second pK of PA, about which the H^+ release was measured, was independent of the concentration of M^{2+} in the solution and of the degree of binding of the cation. Both these assumptions are in disagreement with the findings reported here. Abramson *et al.* [1] calculated apparent binding constants of 1.2×10^4 and 0.97×10^4 M^{-1} for the reactions of Ca^{2+} and Mg^{2+} with PA^{2-} in the presence of 0.1 M TMACl at pH 7.0. The small difference in Mg^{2+} and Ca^{2+} values found, was within the range of variation of the single values from which the average apparent binding constants were calculated.

Reevaluation of the data of Table 1 of that study [1] for pH 7.0 for the concentrations giving 1/4 and 1/2 saturation of the PA with Ca^{2+} gives $K_{app\,(1/4)} = 1.1 \times 10^4$ and $K_{20} = 2.7 \times 10^3$ M^{-1}. The former value is seven times larger than the value obtained by the murexide technique in the present study at pH 8.5 in the presence of 0.3 M NaCl, and it is suggested that at least part of the difference may be due to double layer effects of the added Ca^{2+} in the former study [1].

Ion Specificity of M^{2+} Binding

Table 3 shows that the ion specificity for binding on PA within a dimyristoyl PC matrix is $La^{3+} \gg Mn^{2+} \sim Mg^{2+} > Ca^{2+} > Ba^{2+}$. This is in disagreement with previous studies based on the effect of these cations on surface potential change [18] and H^+ release [1]. However, the present observations are in line with the aqueous phase complexation data of Smith and Alberty [21] who showed a $Mn^{2+} > Mg^{2+} > Ca^{2+}$ specificity for complexation with phosphate^{-2}, as well as for creatine phosphate^{-2}, AMP^{-2}, ADP^{-3} and ATP^{-4}. The complexation constants for Mn^{2+}, Mg^{2+} and Ca^{2+} in 0.2 M $(n-C_3H_7)_4NCl$ are 382 ± 29, 76 ± 3 and 50 ± 2 M^{-1}, respectively.

Extrapolated to zero ionic strength, the Ca^{2+} association constant for HPO_4^{2-} becomes 500 M^{-1} and the corresponding association constant for

Table 3. Specificity of divalent cation binding to dimyristoyl PC/PA membranes

Mole fraction PA	Ion specificity[a] normalized to Ca^{2+}				
	Ba^{2+}	Ca^{2+}	Mg^{2+}	Mn^{2+}	La^{3+}
0.1	0.34 ± 0.10	1.00	0.88 ± 0.25	2.13 ± 0.20	~ 10
0.2	0.48 ± 0.12	1.00	1.56 ± 0.20	1.46 ± 0.20	66 ± 30
0.3	0.53 ± 0.10	1.00	1.75 ± 0.40	1.54 ± 0.30	~ 100
0.5	0.34 ± 0.10	1.00	1.65 ± 0.30	1.34 ± 0.10	~ 100
Average for all mole fractions	0.42 ± 0.07	1.00	1.46 ± 0.18	1.62 ± 0.31	$10-100$

[a] $K_{20\,(M^{2+})}/K_{20\,(Ca^{2+})}$.

The data are the average of at least 6 determinations on 3 different preparations, under the experimental conditions KCl = 0.0 and 9.7 mM, 3.9 mM imidazole buffer, pH 7.3. The $\pm$ values indicate the standard deviation of the mean. The K_3 values for the divalent cations were identical to those of Ca^{2+} for the same mole fraction PA. La^{3+} showed significantly higher K_3 values.

$H_2PO_4^-$ is 12 M^{-1} [5]. These association constants are all within an order of magnitude of the "chemical" association constants, K_{20}, for Ca^{2+} with the phosphate group of PA on the membrane. This taken together with the similarity of the ion specificity ratios indicates that the complexation process on the environment of the membrane surface is similar to that in the aqueous phase.

Modification of the Gouy-Chapman Equations

In the present study, the various ion binding equilibria were treated in terms of a calculated potential to which all ions in the system were considered to respond. In Fig. 6 this potential, $\log(f(\sigma))$, is compared with the potential calculated from the Gouy-Chapman equations as a function of σ and ionic strength of the medium. The difference between the two potentials is large, and increases with increasing surface charge and decreasing ionic strength. This deviation from the ideal Gouy-Chapman behavior will be discussed in terms of discreteness of charge.

The applicability and limitations of the Gouy-Chapman equations have been discussed at length in a large number of publications (*cf.* [6, 10, 20]). The major shortcomings of the theory arise from the fact that it treats the charge on the surface as "smeared out" but treats the ions in the solution as point charges. Furthermore, the effects of finite ionic size or repulsion between the ions in solution are neglected. Application of the Gouy-Chapman theory in unmodified form to the PC/PA membranes would thus

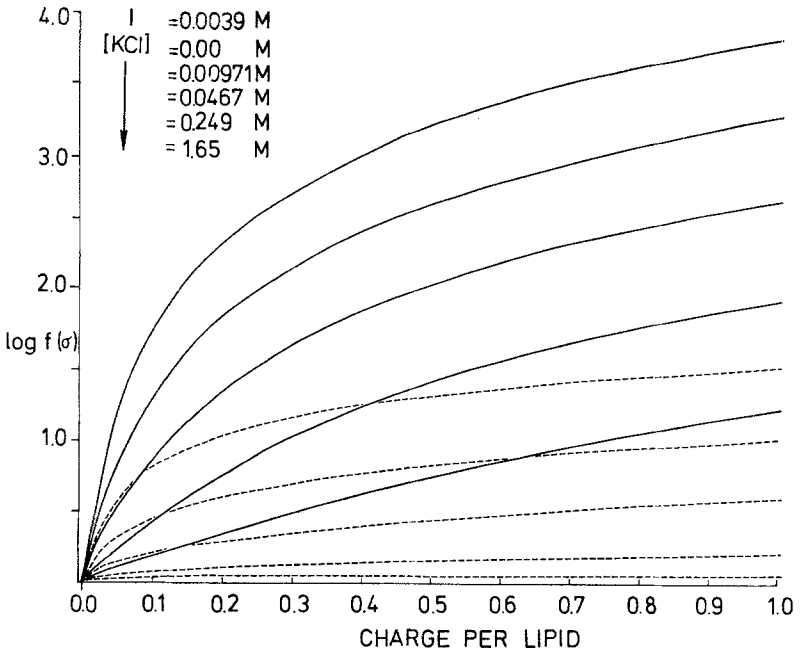

Fig. 6. The dependence of potential on the membrane surface on ionic strength and membrane surface potential. One unit change in $\log(f(\sigma))$ corresponds to a 59-mV change in potential. The calculations were made for molecular areas of 58 Å^2 per lipid molecule, 3.9 mM 1:1 electrolyte (I) and the variable KCl concentrations indicated. The solid lines and the broken lines indicate the dependencies for the Gouy-Chapman equation [Eq. (7)] and the modified Gouy-Chapman theory [Eqs. (7) and (9), with $k_\rho = 103$ M^{-1}], respectively

require us to imagine that the negative charge imparted to the membrane by the PA^- is somehow "smeared" onto the ANS^- binding site composed of four neutral PC polar head groups. We would also be required to assume that the short-range electrostatic interactions between Ca^{2+} and PA^- are adequately described by a model in which the PA^- has lost a large portion of its charge through some process which "smears out" the charge over the region occupied by the PC polar head groups.

The model involving "smearing out" of the membrane surface charge is successful in describing electrostatic interactions at the membrane surface at low ionic strength where $1/\kappa$ (*cf.* [17]) is large compared to the distance between the charges on the membrane surface. However, this is not the case in the system of the present study where application of the Gouy-Chapman theory predicts a large potential drop near the membrane surface. It predicts that the potential can be halved within distances as small as 6 Å normal to the membrane and that 10-fold differences in counter-ion con-

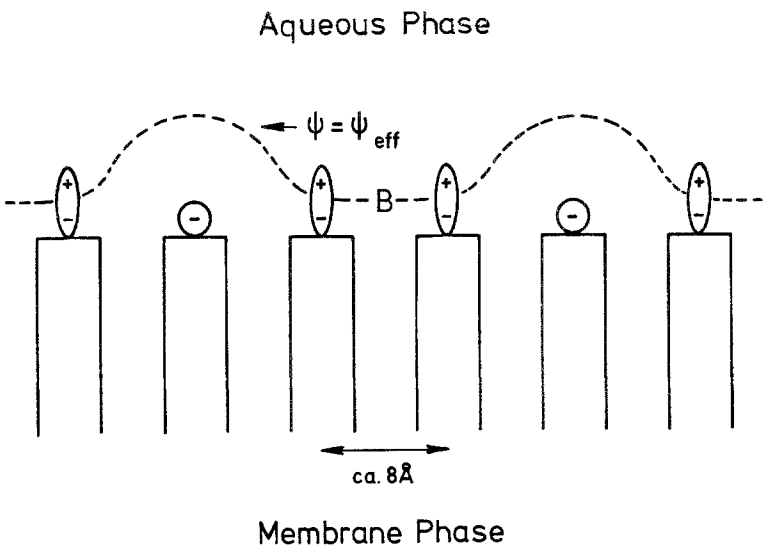

Fig. 7. A schematic representation of potential distribution of the surface of a PC/PA membrane. The stick figures represent PC and PA molecules, and the broken line represents positions where the electrical potential is equal to the effective surface potential, ψ_{eff}, measured for the ANS^- binding site

centration can be expected for distances comparable to an ionic diameter. Since these distances are smaller than the minimum distance between the ANS^- binding site and the PA^- molecules (ca. 12 Å) it seems unlikely that all microregions of the membrane surface could be described by a unitary surface potential.

An alternative interpretation is offered in Fig. 7. The broken line represents positions where the potential is equal to an effective surface potential ψ_{eff} at the ANS^- binding site, determined from the experimental $f(\sigma)$ values. The ψ_{eff} isopotential intersects the ANS^- binding site (B) but may extend several Å into the solution in the region of membrane surface charges. This representation differs from that of the Gouy-Chapman theory which predicts that all isopotentials are parallel to the membrane. The region of the aqueous phase up to the ψ_{eff} isopotential is considered to obey the Gouy-Chapman equation for potential and counter-ion concentration distribution. Any electrostatic interactions occurring in the region between the ψ_{eff} isopotential and the membrane surface are considered to be characteristic for the geometry of the system and independent of the electrolyte composition of the aqueous medium.

Reference to Fig. 7 indicates that the "chemical" binding constant K_{20} may contain a residual electrostatic component inasmuch as the correspond-

ing process involves moving Ca^{2+} from the ψ_{eff} isopotential to its complexing position on the PA^-. Table 1 shows that K_{20} shows at most a sevenfold variation, indicating that this residual electrostatic component can vary no more than 20 mV with extreme variations of membrane surface charge and electrolyte composition of the medium.

The interpretation of Fig. 7 is supported by several studies which have shown that the effect of discreteness of charge can result in potentials on the membrane surface which are smaller than those which would be calculated using a model in which the charges are "smeared out" [4, 9, 10]. At high values of surface charge, the inner portion of the electric double layer can be represented by two parallel sheets containing hexagonal arrays of oppositely-charged ions [9]. This is the approximate situation when an array of PA^- molecules dispersed in a lecithin matrix attracts a layer of counter-ions. Grahame [9] has made calculations for the "microscopic" surface potential ψ_a at a point where one of these surface charges has been removed. This can be compared directly with the effective surface potential ψ_{eff} measured here and represented in Fig. 7. Grahame [9] compared ψ_a with the "macroscopic" potential ψ calculated on the assumptions of "smeared out" charge. The relationship between these two potentials is given approximately as $\psi_a/\psi = 0.766*(d/r) - 0.67*(d/r)^3$ where r is the distance between the fixed charges on the membrane surface and d is the distance between the two planes.

The discrepancy between the "macroscopic" and "microscopic" potentials can be evaluated using estimates of d and r. For the high surface charges of the present system, the Gouy-Chapman theory indicates a steep drop in potential near the membrane surface, corresponding to d values as low as 4 Å. This value, when used together with $r = 8$ Å as the approximate lower limit for the distance between charged centers (the PA^- charges or the PA^- charges and the ANS^- binding sites) predicts $\psi_a/\psi = 0.295$. Since the inner portion of the double layer accounts for the greatest portion of the potential drop, this calculation shows that the Gouy-Chapman equations can seriously overestimate the potential affecting ion binding reactions such as those studied here, offering additional justification for the modification of the equations.

Comparison with Other Techniques

Macroscopic surface potential, averaged over large areas, can be determined by the air electrode (*cf.* [12, 18]). The method has the advantage that it measures both the surface charge and surface dipole contributions, but has the serious disadvantage that its use is limited to monolayer systems.

Electrophoretic methods can be used to determine the zeta potential, defined as the potential at that distance from the membrane where the solvent water is free to slip upon movement of the membrane. Calculation of the surface potential from the zeta potential requires that assumptions be made about the position of the slip plane. The application of both methods to problems of ion binding requires that discrete charge effects be either negligible or neglected.

A method of surface potential determination similar to that of the present study uses the shift in pK umbelliferone, an extrinsically added hydrophobic pH indicator [16]. In this study, electrostatic potential values were determined in detergent micelles and in phospholipid membranes. The potentials measured agreed with the zeta potentials of these systems.

Haydon and Meyers [12] have studied the variation of nonactin-induced K^+ conductance in bilayer membranes in which the surface charge was changed by the absorption of small amounts of ionic detergents. These conductance changes were compared with the total potential changes across the membrane interface as inferred from compensation potential changes in oil- and monolayer-aqueous interfaces. The conductance changes found were almost totally accounted for by changes in the surface potential calculated from the Gouy-Chapman theory, and it was concluded that surface dipole effects contributed to the differences observed. The bilayer study [12] was conducted for low surface charges, with one charge per 775 $Å^2$ or more, under conditions where discreteness of charge effects were negligible.

McLaughlin *et al.* [15] have used the iodide and nonactin-induced K^+ transport reactions to measure the surface potentials of phosphatidyl serine and phosphatidyl glycerol lipid bilayer membranes. In this study [15], conducted at one monovalent cation concentration ([KCl] = 0.1 M), it was concluded that the differences in membrane conductance in charged (PS^- and PG^-) membranes and uncharged membranes (PE) were due to differences in the membrane surface potential. Using the Gouy-Chapman theory it was calculated that the PG and PS membranes had molecular areas of 39 Å per lipid molecule (i.e. area per charge). It has been pointed out by Haydon and Hladky [11] that this molecular area is "prohibitively low", and it is emphasized here that this has a serious effect on the validity of the conclusions drawn. The effects of added Ba^{2+} and Sr^{2+} in PG and PS bilayers [15] were in accordance with the expectations of the Gouy-Chapman theory using 39 $Å^2$ per lipid molecule, leading to the conclusion that these ions do not bind to these membranes. The effects of Ca^{2+} and Mg^{2+} on PS bilayers were larger than expected from the Gouy-Chapman theory and this was taken as evidence for direct binding of these two species. However, the

binding constants were very low ($K_{20} \approx 0.1$ M^{-1}). It is worth pointing out that had the more probable value around 58 Å^2 ([3, 22], *cf.* [11]) been used in the analysis, the calculations would have indicated that all four divalent cations bound, with binding constants appreciably larger than those reported. A study of the interactions of Ca^{2+} with membranes containing PS and PG using the ANS^- and murexide techniques may indicate whether those differences are due to differences on the experimental systems or differences in interpretation.

Conclusions

The most important finding of the present study is that ANS^- can be used as a fluorescent nonperturbing indicator of electrical potential near the membrane surface in model and biological membranes. The exponential function of membrane surface potential at the ANS^- binding site, $f(\sigma)$, can be simply calculated as K_1/K_{10}, where K_{10} is evaluated at [MCl] = 1.0 M. This is possible because the probe binds in the anionic form and does not ion pair with added cations.

The membrane surface charge σ, and hence the degrees of cation binding, can be calculated from the dependence of $f(\sigma)$ on the ionic composition of the medium. This dependence can either be determined as the empirical relationship established in experiments in which σ is varied, or can be calculated using the Gouy-Chapman equations under conditions in which they are appropriate. The course chosen in the present study was to modify empirically the Gouy-Chapman equations to obtain the experimentally-determined dependence of $f(\sigma)$ on σ and the ionic composition of the medium. This is equivalent to using an empirical relationship to determine σ.

Compared with other methods for determination of surface potential effects, the ANS^- method has distinct advantages. The method can be used over a large range of pH and the measurement requires no pH perturbation. Furthermore, the method can be applied to intact tissues such as muscle and nerve, and the fluorescence signal provides instantaneous and continuous measurement of potential charges at the membrane surface. The binding reaction of ANS^- to phospholipid membranes is fast [13] with binding and dissociation constants of 2.1×10^8 M^{-1} sec^{-1} and 4.5×10^3 sec^{-1}, respectively [13], indicating that it should be capable of reporting the kinetics of surface potential changes for times greater than 200 μsec.

One possible drawback to the use of the ANS^- method for determining surface potential and changes in surface charge is the possibility that changes in the molecular organization of the membrane with external conditions affect the ANS binding. An example of this is the effect of Ca^{2+} binding on

the distribution of PA within lecithin membranes affecting the number of four-membered binding sites for ANS^-. Furthermore, the type of binding site analysis given here will be difficult to carry out with many biological membrane preparations. However, electrostatic and structural effects on ANS^- binding can be separated from each other by comparison with the behavior of a cationic probe of the same binding site. Reversal of the sign of the response will indicate that the effect is primarily electrostatic. Alternatively, the degree of M^+ or M^{2+} binding to membranes reported by ANS^- can be checked with an aqueous indicator, as was done here. In the most unfavorable cases, ANS^- may be considered only as a qualitative indicator of membrane surface potential.

The present study has shown that divalent cations react with PA^- to form a 1:1 complex with a $Mn^{2+} \sim Mg^{2+} > Ca^{2+} > Ba^{2+}$ specificity. The equilibrium constants for these reactions are the product of "chemical" (K_{20}) and electrostatic ($f(\sigma)^{-2}$) contributions. The K_{20} value for Ca^{2+}, which contains a sizable but essentially constant electrostatic contribution, is only one order of magnitude larger than the association constant for Ca^{2+} and $H_2PO_4^-$ in solution, which also contains a sizable electrostatic contribution. The similarity in the values indicates the Ca^{2+} is bound on the membrane in an essentially aqueous environment. Evidence has been found that the 1:1 complex can attract a second PA^-, but formation of a 1:2 complex does not seem to be obligatory and there is no evidence for formation of a separate phase upon Ca^{2+} binding.

The apparent binding constant for M^{2+} increases with decreasing ionic strength and increasing negative surface charge, due to a corresponding change in $f(\sigma)^{-2}$.

The potential to which ANS^- and M^{2+} binding respond is smaller than that predicted by the Gouy-Chapman theory. However, this potential could be calculated using the Gouy-Chapman theory with a modification which allowed a portion of the electrostatic energy of reaction of the ions with their binding sites to be dependent on the position of these binding sites relative to the other fixed charges in the system. The value of this residual electrostatic energy is characteristic for the specific reaction under consideration and is fairly constant with ionic strength.

It is concluded from considerations of the effects of discreteness of charge that the membrane surface of phospholipid mixtures cannot be characterized by a single homogeneous potential and that different values of membrane surface potential will be obtained when different methods are applied. Our measured value of the membrane surface potential will be dependent upon our position in time and space [7].

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