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ELECTROSTATIC INTERACTIONS AT CHARGED LIPID MEMBRANES. CALCIUM BINDING *

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A quantitative study of calcium-ion binding by the negatively-charged phospholipid methylphosphatidic acid is presented. Experimental results are compared with the predictions of the Gouy-Chapman theory, taking into account both the ions bound at the membrane surface and the ions held in the diffuse layer. This theory suffices to explain the titration of the calcium/lipid system, but fails to explain completely the behaviour of the ordered-fluid transition temperature, which shows a splitting that according to electrostatic theory alone should not occur. The dependence of the calcium-lipid binding constant upon 1 : 1 electrolyte concentration is correctly predicted by the theory; the latter however gives equations which can only be solved numerically. A simple, approximate equation is therefore given (in the text, eq. 34) for the prediction of the degree of calcium binding to a negatively-charged lipid membrane.

1. Introduction

In the first paper in this series [1] an expression was derived for the electrostatic free energy of a charged lipid membrane, and its validity was investigated using the crystal-liquid crystal ("ordered-fluid") phase transition of the membrane; the lipid used was the methyl mono-ester of phosphatidic acid (MPA; fig. 1). This is a particularly suitable lipid for such an investigation on account both of its small polar head-group, which reduces complicating effects due to interaction between neighbouring head-groups, and of its mono-basicity, which simplifies its behaviour in titration.

It was found that the dependence of the phase transition temperature upon pH and upon salt concentration could be explained adequately using the expression derived in ref. [1], which utilised (i) the concept of the work done in changing up the membrane without its chemical composition, and (ii) the simple Gouy–Chapman theory of the double layer. The theoretical background for this has been discussed in detail [2].

It was thus possible to describe in very simple terms the pattern of proton binding to lipid membranes composed of MPA, and other lipids with simple, protonatable head-groups show at least qualitatively similar behaviour (e.g., [3,4])**. No binding of salt was observed except for a small, apparently ion-specific effect of concentrated univalent cations upon the phase transition of MPA [1]. In the case of divalent ions a specific binding is more to be expected, not only because divalent cations have in general a stronger affinity for anions, but also because any enhancement in ion

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** In the meantime further studies [19] have shown that the picture can be made more complex by hydrogen bonding within the surface of the membrane.

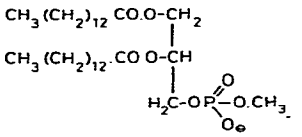

Fig. 1. Methylphosphatidic acid (MPA). Under all conditions described in this paper the phosphate group was deprotonated, as shown here.

binding caused by electrostatic factors would be squared, owing to the exponential term in the Boltzmann law. A further complication is that the divalent ions, unlike protons, are present at a significant concentration in the diffuse layer and contribute to the screening of the negative charge present at the membrane surface [5]. In consequence the Gouy–Chapman equations become more complicated and only a numerical solution is possible.

We describe in this paper investigations directed towards determining the degree of calcium ion binding at the surface of fully deprotonated MPA, along with a comparison between results thus obtained and the predictions of the simple Gouy–Chapman theory. Previous work has already shown that this simple theory provides a correct empirical relationship between the charge density on a lipid membrane and its surface potential [5,6,7] and phase transition temperature [1,8] when only univalent ions are present; a good correlation with surface potential has also been found for some divalent cations [5].

2. Theory

2.1. Metal binding and the diffuse layer

We consider a lipid membrane with a charge density of σ_0, in the presence of divalent and univalent cations and of univalent anions. σ_0 is equal to e/f, where e is the electronic charge and f the area of a lipid molecule in the membrane; the membrane is fully deprotonated. The charge on the surface is neutralised by the sum of four contributions: (i) divalent ions bound specifically to the membrane surface: (ii) accumulation of divalent cations in the diffuse layer, in excess of their bulk concentrations; (iii) similar accumulation of univalent cations in the diffuse layer; (iv) expulsion of univalent anions from the diffuse layer. There is no specific binding to the membrane of univalent ions, but the divalent cations have an intrinsic affinity for the headgroups of the lipid, defined in the absence of electrostatic effects by the binding constant K. We shall use σ to denote the surface density of charge after partial neutralisation by specific binding of calcium, the surface potential is denoted by ψ_0 and the concentrations of ions with charge 2+, 1+ and 1− by C_{++}, C_+ and C_- respectively. Thus for the reaction

$$Ca^{2+}_{(free)} + 2L^- \rightarrow CaL_2$$

the binding constant will be raised by a factor $\exp(2e\psi_0/kT)$, so that

$$C_{++} K \exp(2e\psi_0/kT) = (\sigma_0 - \sigma)/\sigma\,, \qquad (1)$$

whence

$$\sigma = \frac{\sigma_0}{1 + KC_{++}\exp(2e\psi_0/kT)}\,. \qquad (2)$$

It is also convenient to define a binding fraction f_b, equal to the ratio of bound calcium ions to lipid molecules in the membrane. It is apparent that

$$f_b = \tfrac{1}{2}(1 - \sigma/\sigma_0)\,. \qquad (3)$$

In writing eq. (1) the implicit assumption was made that saturation (full neutralisation) of the membrane with bound calcium results in occupation of all the binding sites available for calcium, as is usually the case in complexation reactions. This is however not true. Although the nature of the sites on which bound calcium ions reside is not known (i.e., bound to one phosphate group, or chelated by two or three such groups, with a conceivable co-ordination number between one and six), it is clear that the number of available sites may be greater than the number of bound calcium ions at saturation. Thus each incoming ion blocks more than one potential binding side for the next ion, causing a reduction in the affinity of the membrane for calcium as the fraction of neutralised lipid groups increases. Consequently the "constant" K will vary. So far only a one-dimensional solution for this problem has been put forward [9]; a solution for the calcium-lipid system will only be possible when the nature of the calcium-binding site is known.

The Gouy–Chapman theory has been outlined in reference [1] and more detailed discussion of it may be found in references [10] and [11]. Here we will simply make use of its results. In the presence of a mix-

ture of ions a membrane of surface charge density σ experiences a surface potential ψ_0 given by

$$\sigma = 2\epsilon_r\epsilon_0 kT \sum_i (C_i[\exp(z_i e\psi_0/kT) - 1])^{1/2} \quad (4)$$

where e is the electronic charge, ϵ_0 the permittivity of free space, ϵ_r the dielectric constant and z_i the charge number of the ith ion. S.I. units are used here and in the calculations which follow. The concentrations in eq. (4) refer to the bulk phase, where the potential is zero. The concentration of the ith ion at a distance x from the membrane, where the potential is ψ_x, is given by

$$C_i(x) = C_i \exp(z_i e\psi_x/kT) . \quad (5)$$

To find ψ_x it is necessary to take a second equation from the Gouy–Chapman theory, in fact the one used to derive eq. (4), namely

$$\frac{d\psi_x}{dx} = -\left(\frac{2kT}{\epsilon_r\epsilon_0}\sum_i C_i[\exp(z_i e\psi_x/kT) - 1]\right)^{1/2} . \quad (6)$$

The procedure for finding the distribution of ions around the membrane is straightforward. First eqs. (2) and (4) are combined so as to eliminate σ. Since σ_0 is known, ψ_0, the only remaining unknown, may be found by iteration. (It is of course necessary to insert a value for K, since the chemical origins of this parameter lie outside the scope of the Gouy–Chapman treatment.) Substitution of ψ_0 into eq. (2) gives σ, which leads via eq. (3) to f_b.

The excess of ions in the diffuse layer at a distance x from the membrane, obtained from eq. (5), is

$$C_i(x) - C_i = C_i(\exp(z_i e\psi_x/kT) - 1) \quad (7)$$

and the accumulation of ions in the entire double layer, expressed as the amount of charge per unit area that they represent, is therefore given by the screening integral

$$S_i = z_i C_i \int_0^\infty (\exp(z_i e\psi_x/kT) - 1)\, dx . \quad (8)$$

This may be integrated if eq. (6) is used to express dx in terms of ψ_x and $d\psi_x$. The resulting expressions are

$$S_{++} = 2C_{++} \int_{\psi_0}^{0} \frac{e^{2q} - 1}{r} d\psi_x \quad (9)$$

$$S_+ = C_+ \int_{\psi_0}^{0} \frac{e^{q} - 1}{r} d\psi_x \quad (10)$$

$$S_- = -(C_+ + 2C_{++}) \int_{\psi_0}^{0} \frac{e^{-q} - 1}{r} d\psi_x \quad (11)$$

where

$$q = q(x) = e\psi_x/kT \quad (12)$$

and

$$r = r(x) = [2kT/\epsilon_r\epsilon_0]^{1/2} \times \{C_+(e^q - 1) + C_{++}(e^{2q} - 1) + (C_+ + 2C_{++})(e^{-q} - 1)\}^{1/2} . \quad (13)$$

To express the concentrations in mole/litre, a further factor of 6.023×10^{26} is inserted before the factor kT in eq. (13). Since the screening terms together neutralise the membrane left unneutralised by bound calcium, the integration can be checked using relation (14).

$$\sigma/e = S_{++} + S_+ + S_- \quad (14)$$

Finally, in a titration experiment it is not possible to distinguish between calcium ions which are bound to the surface and those which are trapped in the diffuse layer. The number of calcium ions thus trapped contributes to the total number of calcium ions taken up by the membrane, which is therefore given by

$$f_t = f_b + \tfrac{1}{2} f S_{++} . \quad (15)$$

2.2. The phase transition temperature

The basic equations derived in ref. [1] which relate the changes in phase transition temperature to surface electrostatic effects are

$$T_t = T_t^* + \Delta G^{el}/\Delta S^* \quad (16)$$

$$\Delta G^{el} = L(\phi + f\, d\phi/df)\Delta f \quad (17)$$

$$G^{el}/Lf = \phi = \int_0^\sigma \psi_0(\sigma')\, d\sigma' . \quad (18)$$

Here T_t is the transition temperature and T_t^* that of the neutral lipid. G^{el} is the electrostatic contribution to the Gibbs' free energy of the charged lipid membrane

(per mole of lipid) and ϕ is the same quantity expressed per unit area. L is Avogadro's Number and f the area occupied by one lipid molecule in the membrane. Δf, ΔG^{el} and ΔS^* are changes at the transition; S^* is the molar entropy of the neutral lipid.

When only univalent ions are present, an explicit expression for ψ_0 can be obtained, so that integration of eq. (18) and substitution of the result into eqs. (17) and (16) is possible. In the presence of a mixed electrolyte one must proceed differently. Since $\sigma = e/f$, $d\sigma = -(\sigma/f)\,df$. Use of this to differentiate ϕ (eq. (18)) with respect to f, and substitution of the result into eqs. (17) and (16) yields

$$T_t - T_t^* = \frac{\Delta G^{el}}{\Delta S^*} = -\frac{L\,\Delta f}{\Delta S^*} \int_0^{\psi_0} \sigma(\psi_0')\,d\psi_0' \,. \qquad (19)$$

Since we have σ as a function of ψ_0 it is possible to integrate numerically, and applying known values of Δf, ΔS^* and T_t^* leads via eq. (16) to a prediction of T_t.

Before comparing the results of such calculations with experiment, some comments on the form of the results to be expected are pertinent. (i) The selective advantage of calcium enrichment (over sodium enrichment) in the double layer is proportional to $\exp(2e\psi_x/kT)/\exp(e\psi_x/kT)$, i.e., to $\exp(e\psi_x/kT)$. Consequently a low surface potential – brought about by a high 1 : 1 electrolyte concentration – will minimise the amount of calcium in the diffuse layer. If this amount is negligible, then once eqs. (2) and (4) have been solved the system can be treated analytically, with the use of eqs. (16) to (18) leading to an explicit expression for T_t, as was obtained for the case of partial neutralisation by protons in ref. [1]. As will be seen, this is a reasonable approximation for physiological conditions. (ii) In consequence of the reduction in surface charge density accompanying progressive neutralisation of the membrane, the strength of binding decreases as more calcium is bound. In fact, making the approximation mentioned above, it is easy to show that the slope of the titration curve of a membrane at the halfway point is equal to $(4+4n)^{-1}$, where n is the charge number on the cation being bound and zero for neutral species. This means that while a membrane is a worse buffer of its environment than is a simple molecule in which the ion-binding sites do not interact, its degree of dissociation is conversely less sensitive to the surrounding medium than is that of the simple molecule. This is illustrated in fig. 2.

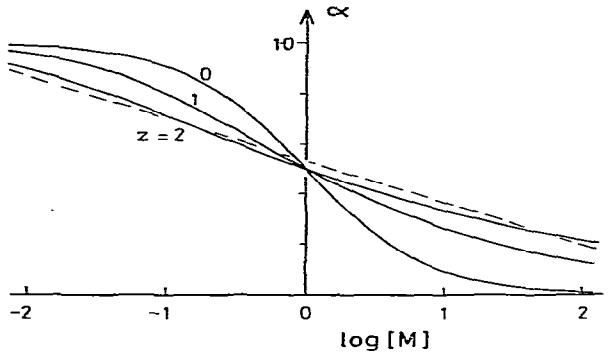

Fig. 2. Theoretical curves for titration of a membrane with a species M of charge z. They are given by $M = (1-\alpha)/\alpha^{(2z+1)}$. The dotted line represents a linear approximation to the curve for $z = 2$. Arbitrary horizontal displacement.

3. Experimental

Binding measurements were carried out using filtration or centrifugation combined with chemical analysis, or with the calcium ion electrode. A programmable calculator (Hewlett-Packard 67) sufficed to carry out the numerical integrations.

3.1. Materials

MPA was synthesized according to published procedures by Dr. H. Eibl (e.g. [1]). All salts used were of p.a. quality. Lipid dispersions were prepared by incubation of the pure sodium salt of MPA with distilled water for one hour at 70°C. After mixing with salt solutions and appropriate dilution, the dispersions were incubated briefly at 70°C and then for 12 hours at the appropriate temperature before measurement. Checks on the pH revealed values above 7.5, indicating that the lipid was fully deprotonated. It was further shown that incubation overnight in the presence of calcium at pH 8 and 70°C (i.e., the most rigorous conditions to which the lipid was exposed) did not give rise to decomposition products observable by thin-layer chromatography. With the exception of the experiment shown in fig. 5 none of the dispersions were sonicated.

3.2. Analysis

Calcium concentrations were measured with a Pye

Unicam SP 90B Series 2 atomic absorption spectrophotometer. By calibration after each measurement an accuracy better than 10% was obtained (30% for concentrations below 10^{-5} M; lower limit 10^{-6} M).

Phosphate analysis, used to determine concentrations of MPA, was carried out by Mrs. A. Augustin using the method of Eibl and Lands [12]. The accuracy was 1–2%.

3.3. Indicator

Murexide (Merck) was employed as a metal indicator. It was checked that MPA has no effect upon the absorption spectrum of murexide and murexide had no effect upon the transition temperature of MPA under the conditions of measurement (pH 8, [murexide] = 5×10^{-5} M, [MPA] = 10^{-4} M).

3.4. Centrifugation

Dispersions were centrifuged at 5°C and 20°C in a thermostatted Beckmann Ultracentrifuge Model L5-65 at 50000 r.p.m. Calcium and phosphate concentrations were measured in the supernatant after centrifugation and checked in the uncentrifuged dispersion.

3.5. Filtration

This was used above the upper operating temperature of the centrifuge (40°C). Various ultrafiltration membranes were tested and the most satisfactory, the Amicon PM10, was used at 55°C and 75°C in conjunction with an ultrafiltration cell by the same manufacturers, for which a thermostatted jacket was specially constructed. The incubated dispersion was placed in the ultrafiltration cell and stirred for a further 10 minutes. A few drops of liquid were filtered through the membrane under compressed nitrogen and then stirring was continued for a further 5 minutes. Finally N_2 pressure was reapplied and 4 ml. filtrate passed, of which the first 1 ml. was not collected. The filtration of each sample lasted ca. 30 minutes using a nitrogen pressure of 5 atm.; approximately 90% of the lipid was retained by the filter, and experiments without lipid showed that some of the calcium was lost by binding to the filter. Since this amount was very variable (<10%), no correction was made for it.

3.6. Determination of the degree of binding

The total amount of calcium taken up by the membrane is denoted by f_t. The empirical definition in eq. (20) corresponds to the theoretical one in eq. (15).

$$f_t = [Ca]_{\text{taken up}} / [MPA]_{\text{total}} \tag{20}$$

If the analytical concentrations of calcium and of lipid in the unfiltered dispersion are $[Ca]_1$ and $[Lip]_1$ and in the filtrate $[Ca]_2$ and $[Lip]_2$, it follows that

$$f_t = \frac{[Ca]_1 - [Ca]_2}{[Lip]_1 - [Lip]_2} . \tag{21}$$

Eq. (21) applies equally well to a centrifugation experiment where the subscript "2" denotes concentrations in the supernatant. Furthermore, it is still accurate when separation by centrifugation or filtration has not been complete; this is clear when one assumes that the separation process has depleted the dispersions of n moles of lipid, which will have taken $(f_t \cdot n)$ moles of calcium with it, so that the ratio of the depletions of calcium and lipid equals f_t. A more rigorous proof has been given by Teubner [13]. Once f_t is known, the concentration of free calcium ions is given by eq. (22):

$$[Ca_{free}] = [Ca]_2 - f_t [Lip]_2 . \tag{22}$$

3.7. Electrode measurements

A thermostatted 7 ml polythene cell was used. Small quantities of calcium chloride solution were pipetted (using Eppendorf micropipettes) or injected (using an Agla microsyringe) into the lipid dispersion in the presence of the required amount of sodium chloride. The solution was stirred vigorously with a magnetic stirring disc between additions; stirring was discontinued to take each EMF reading, and at least half a minute (sometimes several minutes) was required before the reading became steady. The calcium electrode used was the Orion calcium ion-selective electrode; a Radiometer electrode was also tested. The reference was a calomel/KCl electrode.

3.8. Transition temperature measurement

This was carried out as in ref. [1], using N-phenyl naphthylamine (Merck) as a fluorescence indicator and

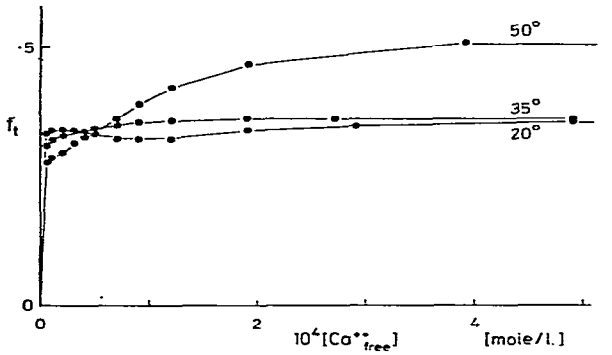

Fig. 3. Titration of MPA with calcium using the calcium electrode. [MPA] = 0.56 m mole/l.; [NaCl] = 10 m mole/l. No further calcium was bound when the free calcium concentration was raised to 7×10^{-4} mole/l.

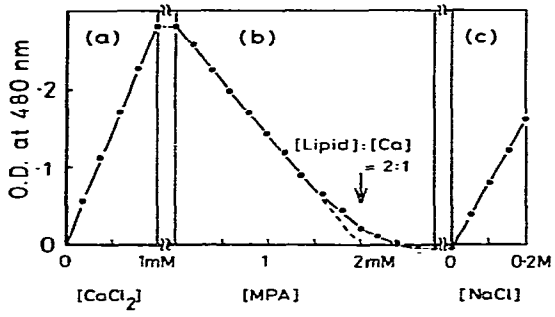

Fig. 4. Murexide titration of Ca^{++} with MPA; the O.D. is measured with respect to a reference cell with the same murexide concentration. [Murexide] = 5×10^{-4} mole/l; [Tris] = 10^{-2} mole/l; pH 8; 20°C. (a) Addition of $CaCl_2$ to the sample cell causes a linear rise in O.D. (b) Addition of MPA to both cells lowers the O.D. untill all the calcium is bound; the extrapolated curve shows the 2 : 1 stoichiometry. (c) Addition of NaCl to both cells reverses the decrease in O.D., since the binding of Ca^{++} to MPA is weakened. The measurements are corrected for dilution.

heating and cooling rates of 1°C per minute. (Slower rates caused no change in the observed fluorescence/temperature curves.) Checks were made using light-scattering as an indicator of the transition.

4. Results and discussion

4.1. Electrode titrations

These were handicapped by the comparative insensitivity of calcium ion electrodes, by their fragility at temperatures above ca. 40°C and by interference apparently due to interaction between the lipid under test and the membrane of the electrode. In consequence, electrode data have not been evaluated quantitatively. Problems were also encountered due to the slow response of the electrode in the presence of lipid and the dependence of the potential observed upon the speed of stirring. The three titration curves shown in fig. 3 were obtained using an Orion electrode; small volumes of calcium chloride solution were added to a dispersion of MPA (0.56 mM) in the presence of 10 mM sodium chloride. It appears that under the conditions of the titration not all of the lipid head-groups are accessible to the calcium ions unless the membrane is in its fluid state (T_t = approximately 47°C for the calcium-saturated membrane, sect. 4.5). Since it was not practical to allow several hours for equilibration between successive additions of calcium, these experiments were not pursued. However the electrode could be used for qualitative observation of "calcium steps" (see sect. 4.4). An electrode by Radiometer was later tested; this promised slightly greater sensitivity and appeared stabler at high temperature. Interaction with the lipid, however, was still present.

4.2. Stoichiometry of calcium binding

It is clear from fig. 3 that the highest attainable value of f_t is 0.5, as expected if one calcium neutralises two MPA molecules. This is also shown clearly by the titration represented in fig. 4a-b, in which murexide was used as indicator. In order to obtain a sharp end-point, conditions were chosen so that the binding of calcium was as strong as possible, i.e., only a small concentration of 1 : 1 electrolyte was present. Addition of sodium chloride reduced the strength of binding, liberating calcium (fig. 4c). The stoichiometry of one mole of calcium to two moles of lipid is again confirmed.

Further support for this is afforded by the behaviour of the phase transition temperature when MPA is titrated with calcium ions at low ionic strength (fig. 5); under these conditions the calcium is bound almost quantitatively, so that the transition temperature (proportional at high surface potentials to the surface density of charge σ – cf. ref. [1]) rises linearly with the addition of calcium until the surface is saturated

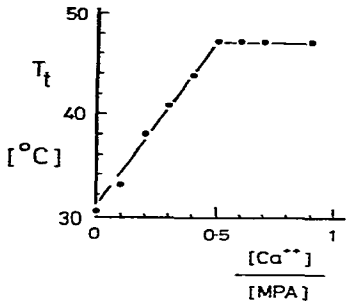

Fig. 5. Phase transition temperature of MPA as function of calcium concentration. The T_t values are taken from phase transition curves measured with rising temperature (e.g. fig. 12). [MPA] = 10^{-4} mole/l, [NPN] = 10^{-6} mole/l, [NaCl] = 10^{-4} mole/l, pH 8. This figure shows only the stoichiometry of the interaction, since this dispersion was sonicated. Sonication does not generally affect the measured T_t but it has a pronounced effect upon the form of the curves (U. Strehlow, personal communication).

at a calcium: lipid ratio of 0.5. The transition temperature for the saturated lipid, where the charge has been neutralised by calcium, is roughly the same as the transition temperature for MPA whose charge has been neutralised by protonation [1]. This behaviour should however be interpreted with caution, since as shown below the behaviour of MPA in the presence at low ionic strength is more complicated.

A calcium: MPA stoichiometry of 1 : 2 has also been found using the change in intensity of the fluorescence of a bound dye [14].

4.3. Binding curves from filtration/centrifugation

Plots of f_t versus $[Ca_{free}]$ are shown in fig. 6. Clearly the accuracy is not high, but the trends observed are those to be expected, viz., (i) f_t varies within the range 0 to 0.5, (ii) binding becomes stronger, the less sodium chloride is present (when no sodium chloride was added, the effective total concentration of sodium ions was 5×10^{-4} mole/l., corresponding to the counterions introduced along with the charged lipid). A close comparison of binding a few degrees above and below the transition temperature was not possible, since in order to avoid complications due to possible phase equilibria "below T_t" must mean below the T_t value of fully-charged MPA, i.e., 26°C, and "above T_t" must mean above the T_t value for calcium-saturated MPA around 47°C.

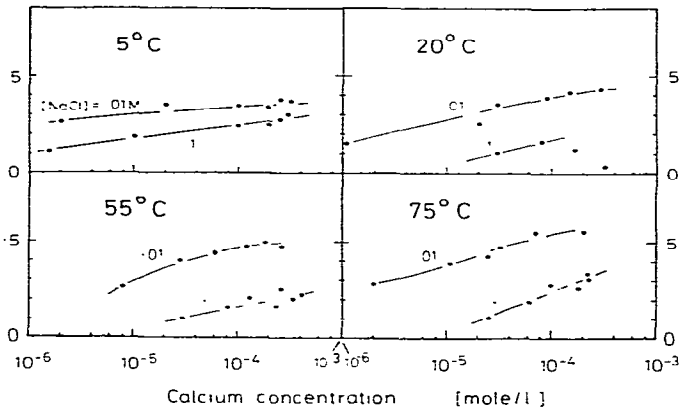

Fig. 6. Degree of calcium uptake f_t as a function of the concentration of free calcium, measured at 0.1 and 0.01 mole/l NaCl by centrifugation (5° and 20°C) or filtration (55° and 75°C).

We can compare these results with the theory on two levels of exactitude, either by ignoring or by taking into account the calcium ions in the diffuse layer. The former treatment has been applied to protons [1], for which it is almost exact, since protons contribute negligibly to the screening in the double layer. The second treatment is exact for all ions, but can only be applied numerically.

4.3.1. "Negligible" calcium concentration in the double layer.

The point of half-equivalence, when $f_t = f_b = \frac{1}{2}$, corresponds to a concentration of free calcium ions, the reciprocal of which defines the apparent calcium binding constant K_{app}. When one mole of calcium is brought up to the membrane surface and allowed to bind, the change in Gibbs' free energy is given by eq. (23), where α is the degree of dissociation ($0 \leqslant \alpha \leqslant 1$).

$$\Delta G = \Delta G^{\text{chemical}} + \Delta G^{\text{el}}$$

$$= \Delta G^0 + RT \ln \frac{[Ca_{bound}]}{[Ca_{free}]\,[Lipid_{free}]} + \Delta G^{\text{el}}$$

$$= -RT \ln K + RT \ln [Ca_{free}] + RT \ln \frac{1-\alpha}{\alpha} - 2Le\psi_0 \qquad (23)$$

However

$$\Delta G = -RT \ln K_{app} - RT \ln [Ca_{free}] + RT \ln \frac{1-\alpha}{\alpha}. \qquad (24)$$

Eq. (24) follows from the definition of K_{app}. Equating the two expressions for ΔG in eqs. (23) and (24), leads to eq. (25)

$$\ln K_{app} = \ln K + 2e\psi_0/kT \quad (25)$$

Since we are dealing with moderately high surface potentials (for discussion see ref. [1]) it is permissable to use the approximation $\sinh x = \frac{1}{2}\exp x$. Consequently we rearrange eq. (4), with $C_{++} \doteq 0$ and $C_+ = C_- = C_\pm$, to obtain

$$\frac{e\psi_0}{kT} = \ln \frac{\sigma^2}{2\epsilon_r \epsilon_0 kT C_\pm}. \quad (26)$$

Combination of eqs. 24, 25 and 26, along with the equilibrium condition $\Delta G = 0$ and the definition $\sigma = e\alpha/f$ ($\alpha = \frac{1}{2}$ at the half-equivalence point) yields eq. (27), and inserting standard values ($e = 1.6 \times 10^{-19}$C, $f = 43 \times 10^{-20}\,m^2$, $\epsilon_r = 8.9 \times 10^{-12}\,kg^{-1}\,m^{-3}\,s^4\,A^2$, $\epsilon_0 = 80$, $k = 1.38 \times 10^{-23}\,JK^{-1}$, $T = 310$ K) gives eq. (28) ($C_\pm$ expressed in mole/l).

$$-\ln C_{++} = \ln \frac{\alpha^5}{1-\alpha} + \ln K + 2 \ln \frac{e^2}{2f^2 \epsilon_r \epsilon_0 kT C_\pm} \quad (27)$$

$$\log K_{app} = \log K + 1.96 - 2 \log C_\pm \quad (28)$$

A similar expression for proton binding has also been obtained [1], viz., $pK = pK_0 + 0.86 - \log C_\pm$. The effect of the 1 : 1 electrolyte in the case of calcium should thus be twice as great as in the case of protons. This is confirmed by inspection of fig. 6. The binding curves measured in the presence of 10^{-2} molar sodium chloride show a half-equivalence point at a calcium concentration of ca. $10^{-5.5}$ mole/l., while the curves measured with 10^{-1} molar salt have half-equivalence points at calcium concentrations around $10^{-3.5}$ mole/l. Thus a tenfold change in the 1 : 1 electrolyte concentration results in a one hundred-fold change in the apparent binding constant K_{app}, as predicted by equation 28. Inserting these values gives $\log K = -0.46$, i.e., $K = 0.35$. This is a reasonable value for the binding constant of a calcium ion to a free anion carrying a single negative charge. However, we have ignored the fact that some of the calcium ions taken up by the membrane are not bound, but are instead trapped in the double layer.

4.3.2. Exact treatment

Here it is necessary to solve the equations for the concentration of calcium at all distances from the membrane surface and to integrate this numerically, as outlined in the theory section above, using eq. (4) to find the surface potential, eq. (9) to determine the amount of calcium accumulated in the double layer and eqs. (3) and (15) to reach a predicted value of f_t. This may be done at various C_{++} values for a fixed C_+ value in order to simulate the binding curves. In each case a value must be assumed for the intrinsic binding constant K. The results of such calculations are displayed in fig. 7, and for comparison with experiment the points from fig. 6 are also plotted. The limitations of the experimental accuracy are made clearer in this plot; even so, it is possible to assert by inspection of the fit that K – the only disposable parameter – has a value around 0.5, in accordance with the above prediction of a value around 0.35. The fit is reasonable for all the curves measured in the presence of 0.1 molar sodium chloride but less satisfactory for those measured in 0.01 molar salt. In the case of the two points where an f_t value of 0.5 is exceeded this is clearly due to experimental error, but in the other cases it is probably connected with the complex behaviour shown by the phase transition of the system in low salt concentrations (see below).

Inspection of the theoretical curves reveals that the f_t is sometimes rather insensitive to the value of K. The reason for this is partly that, as mentioned above, these titration curves are flatter than the titration curves of simple binding systems involving no electrostatic effects, and partly that a compensatory mechanism operates, as follows. If $K = 0$, then the membrane will have its full surface density of charge σ_0 and the surface potential ψ_0 will be high. This enhances the preferential accumulation of calcium ions, rather than sodium ions, in the diffuse layer, making f_t significant even though f_b is zero. If K takes on a finite value, f_b increases, so the surface density of charge σ becomes less than σ_0, and ψ_0 also decreases, so that sodium tends to replace calcium in the diffuse layer as the principal neutralising species. An example of this is given in fig. 8, where under typical conditions K is allowed to increase from zero to a high value. While ψ_0 and f_b change considerably, f_t is not greatly changed. Such behaviour will be seen at all combinations of 1 : 1 and 2 : 1 salt concentrations where the gap between corresponding f_t curves for $K = 0$ and for $K = 5$ in fig. 7 is small. It is thus true, though paradoxical, that in these regions the apparent degree of binding is independent of the binding constant, while the surface potential and charge density vary

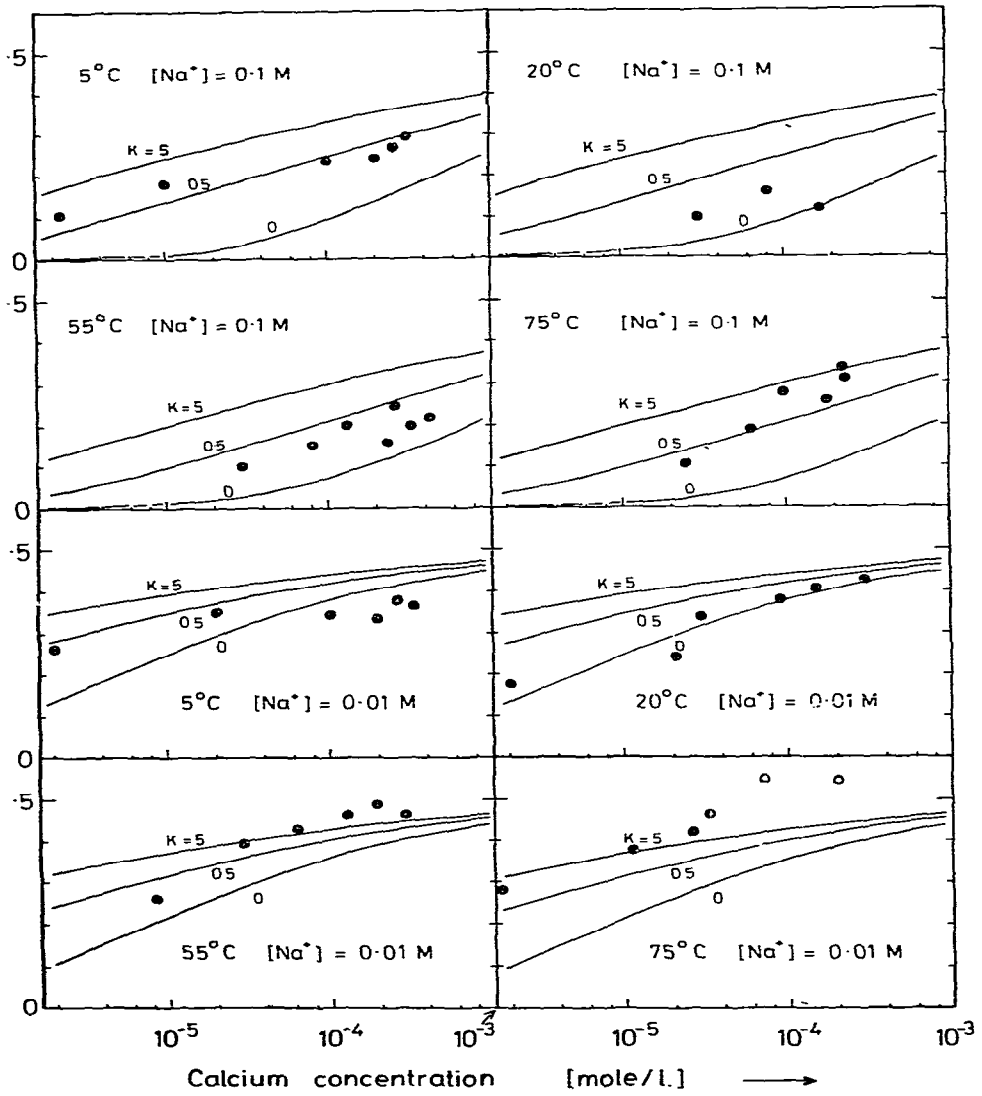

Fig. 7. Theoretical curves for degree of calcium uptake f_t for various temperatures, salt concentrations and K values. Accurate, numerical solution of eqs. (9)–(11) was used to construct these; the molecular area of the lipid was taken to the 40 Å^2 at 5° and 20°C (below T_t) and 46 Å^2 at 55° and 75°C (above T_t). No other, adjustable parameter was used. The points are taken from fig. 6; the two open circles exceed the f_t value of 0.5.

markedly. For this reason, as fig. 8 illustrates, ψ_0 is a more sensitive measure of K than is f_t.

The value of K, the intrinsic binding constant for calcium with MPA in the absence of electrostatic effects, is thus 0.3–0.5 l mole^{-1}. This is similar to the corresponding value of 0.1 l mole^{-1} obtained for magnesium and calcium with phosphatidyl serine using a surface potential measurement [5], and in accord with the values found for alkaline earth ions binding to singly-charged anions which are poor Lewis acids [15]. Lecithins show rather higher binding constants, e.g., $\geq$3 l mole^{-1} [16] (also ref. [17], fig. 14). How-

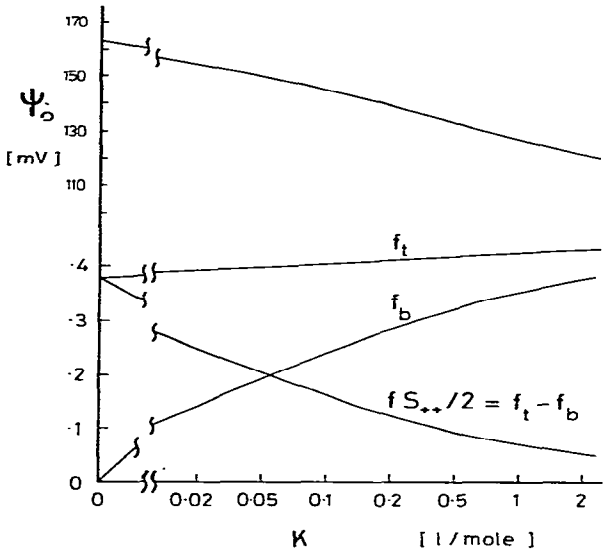

Fig. 8. Illustration of the independence of f_t and K under certain conditions. As K increases (left → right) the surface potential ψ_0 drops, so the increase in f_b is offset by a decrease in S_{++} – cf. eq. (15).

ever, there is increasing evidence [18,19] for the belief that the positive ions in lecithin act more as screening than as binding counterions; consequently the binding of cations will even in this "neutral" lipid be reinforced to some degree by electrostatic effects at the surface. Agreement between K values and binding constants involving free ions has also been found for the system M^{2+}-phosphatidic acid using a charged dye as indicator [20].

The fact that K_0 has the same value below as above the transition temperature suggests strongly that no change in co-ordination number of the metal accompanies the expansion of the membrane at the transition. The low value of K_0 rules out the chelation of the ion by neighbouring phosphate groups.

4.4. Ion "pulses" at the phase transition

The expansion of the lipid membrane at the crystal-to-liquid-crystal phase transition causes a reduction in the surface density of charge and in the surface potential. Consequently the electrostatic enhancement of ion binding is also reduced, so that the apparent binding

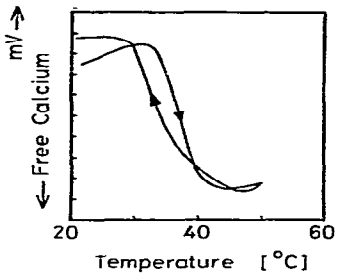

Fig. 9. Release of Ca^{++} from the surface of MPA bilayers at the ordered → fluid transition. The ordinate divisions are 1 mV. [MPA] = 2×10^{-3} mole/l, [NaCl] = 0.2 mole/l.

constant shows a sudden decrease at the phase transition, resulting in the release of bound ions. The consequent step in the ion concentration, both at the membrane surface and in the bulk phase, has already been described for protons [4,13,14] and its magnitude discussed in terms of a possible mechanism for enzyme activation initiated by a change in the physical state of the membrane [21]. A detailed theoretical treatment of this is forthcoming.

For calcium, such concentration steps at the transition are also expected, and these have been observed in preliminary experiments by Träuble [14]; the experimental curve in fig. 9 is reproduced from this reference. We wish here to point out that the approach taken to account for the binding curves above also leads to a satisfactory prediction of the magnitude of the step in calcium concentration measured with the ion electrode. This is a reinterpretation: in ref. [14] the calcium was considered to be quantitatively bound below the transition, and about 1×10^{-4} mole/l. calcium was believed to have been released. However, a full solution is now possible. Using the equations above, it is found that under the conditions of the experiment ([MPA], 2×10^{-3} mole/l; [Ca^{2+}], 2×10^{-4} mole/l; [NaCl], 0.2 mole/l; $T = 310$ K) the concentration of free calcium ions is 1.88×10^{-5} mole/l for the crystalline membrane ($f = 40$ Å^2) and 2.86×10^{-5} mole/l for the liquid-crystalline membrane ($f = 46$ Å^2). The predicted magnitude of the calcium concentration step is therefore 0.98×10^{-5} mole/l. The change in potential at the calcium-specific electrode is given by

$$\Delta E = (RT/nF) \ln (C_2/C_1) = [30.7 \log C_2/C_1]/\text{mV}. \quad (29)$$

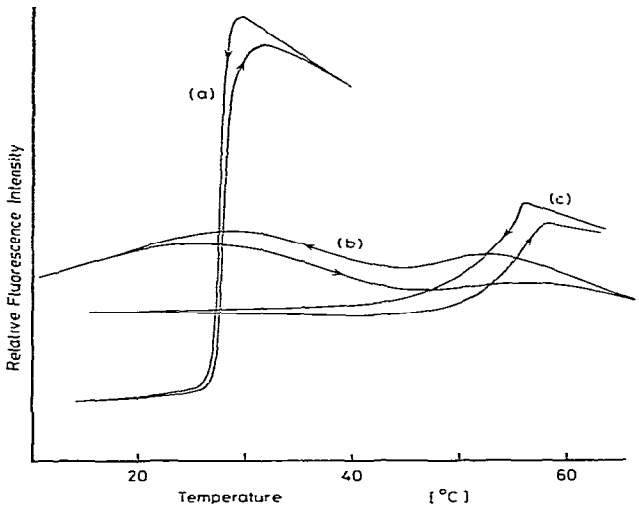

Fig. 10. Splitting of the ordered-fluid phase transition of MPA when it is partly saturated by calcium. [MPA] = 2.5×10^{-4} mole/l, [Tris.$\frac{1}{2}$HCl] = 2×10^{-3} mole/l, [NPN] = 10^{-6} mole/l, [NaCl] = 10^{-2} mole/l. (a) no calcium. (b) [$CaCl_2$] = 1.25×10^{-3} mole/l. (c) [$CaCl_2$] = 5×10^{-3} mole/l.

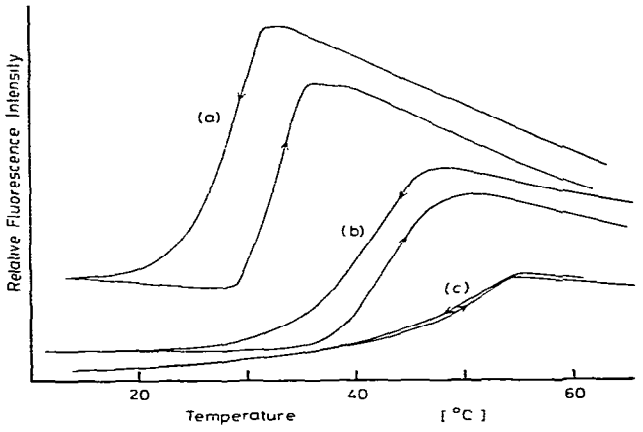

Fig. 11. Absence of splitting of the phase transition of MPA by calcium in the presence of higher 1 : 1 electrolyte concentrations. [MPA] = 2.5×10^{-4} mole/l, [NaCl] = 0.2 mole/l, [Tris] = 8×10^{-4} mole/l, pH 8. (a) no calcium. (b) [$CaCl_2$] = 10^{-2} mole/l. (c) [$CaCl_2$] = 1 mole/l. The decrease in fluorescence intensity during each measurement is due to photolysis of the NPN. Note the much higher calcium concentrations required for saturation than at low 1 : 1 electrolyte concentration (fig. 10).

Putting $C_2/C_1 = 2.86/1.88 = 1.52$, one expects a ΔE value of 5.6 mV, in satisfactory agreement with the 7.2 mV observed; the latter change in emf corresponds to a concentration change by a factor of 1.72. It thus appears that, although the binding curves measured by filtration and centrifugation are not sufficiently accurate to show the small differences between the affinities of MPA for calcium ions in its fluid and ordered states, these small differences can be observed using the electrode, and their magnitude is also in correspondence with the theory.

In general, if the membrane is buffering the calcium concentration, as occurs when membrane-bound calcium is in excess over other forms, then a change in the salt concentration $C_{\pm}$ will cause a change in bulk calcium concentration C_{++} given (from eq. (28)) by

$$\Delta \log C_{++} = -2\Delta \log C_{\pm} \,. \qquad (30)$$

4.5. The crystal-liquid crystal phase transition temperature

Up to now we have made two explicit assumptions in our description of the system. (i) The Gouy–Chapman theory provides a complete description of the diffuse layer. References has already been made to the shortcomings of this assumption (point ions, smeared-out surface charge, bulk-phase value for the dielectric constant), and we have been content to ignore these, using the Gouy–Chapman expressions as an empirical, numerical relation between surface charge density and surface potential, giving only a qualitative physical picture of the structure of the diffuse layer. (ii) In addition to this an intrinsic binding has been assumed between calcium ions and the phosphate groups of MPA, described by a conventional binding constant; the head-groups are thus assumed to provide independent binding sites for the calcium ions. Taken together, these assumptions lead to the conclusion that the membrane surface will always be homogeneous, carrying a distribution of calcium ions which on account of their mutual electrostatic repulsion adopt a distribution even more homogeneous than a random one. The separation of the ions into calcium-rich and calcium-deficient zones would be an energetically unfavourable process and should not take place without the imposition onto the system of some further factor. (This point is argued in more detail in refs. [1] and [14].)

We now come to an effect which cannot be explained

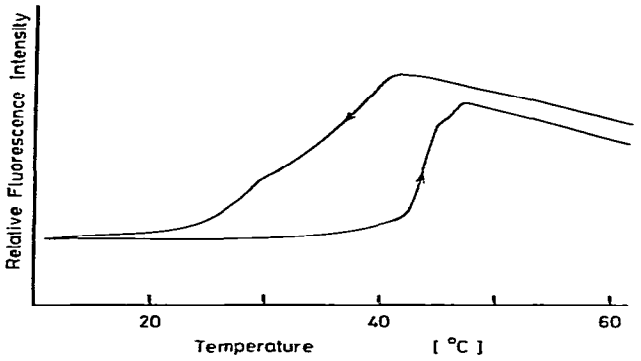

Fig. 12. Intermediate situation seen at intermediate 1 : 1 electrolyte concentrations: a single transition is seen with rising temperature and two with falling temperature. Cf. figs. 10 and 11.

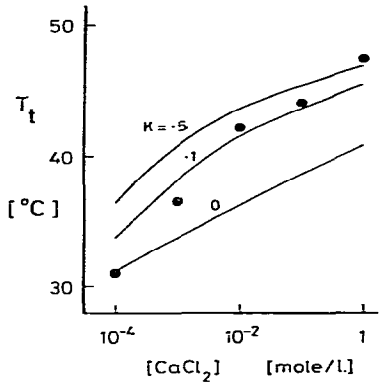

Fig. 13. Theoretical and observed transition temperatures for MPA in the presence of 0.2 mole/l NaCl and varying concentrations of $CaCl_2$. Conditions as in fig. 11.

on this simple basis. It has been described in previous publications [4,14]. This is the splitting of the phase transition curve of MPA when less than the stoichiometric amount of calcium has been bound to it, illustrated in fig. 10. The usual interpretation placed on this is that the calcium ions do not, as predicted in the previous paragraph, become distributed homogeneously over the MPA surface, but instead from "clusters", i.e., calcium-rich patches, on the surface of the lipid. Progressive addition of calcium ions increases the size and/or number of these clusters and reduces the remaining calcium-free or calcium-deficient area, while (by the phase rule) the concentration of free calcium in solution remains constant.

By carrying out titrations of MPA with calcium we have shown clearly that the splitting of the phase transition curve is related to the 1 : 1 electrolyte present. Our experimental data may be summarised by the generalisation: in the presence of 1 : 1 electrolyte in a concentration of 0.1 mole/l or above, no splitting of the transition curve is seen. Instead, the single transition temperature rises monotonically from the value corresponding to the negative lipid to that corresponding to the neutral lipid (fig. 11). At 1 : 1 electrolyte concentrations at or below 10 mole/l the phase transition curve is clearly split, as shown in fig. 10. At intermediate concentrations of 1 : 1 salt no clear behaviour is seen; the hysteresis shown by the transition curves becomes very large, and the two transition temperatures merge into one another. Sometimes a single transition is seen with rising temperature and two with falling temperature (fig. 12); there appears to be some connection between this phenomenon and the previous treatment of the system (sonication, incubation, etc.). Such behaviour has already been seen, shown by the system magnesium-phosphatidic acid [4], but the dependence upon bulk salt concentration was not at the time appreciated. The effect of Li^+ and of Cs^+ at 30 mM was the same as that of Na^+; no ion specificity could be seen.

There is thus an apparent co-operativity in the binding of calcium to MPA at low salt concentrations which disappears when the salt concentration is raised. Some possible origins of this will be discussed.

4.5.1. Behaviour at high salt concentration

Fig. 13 shows the effect of calcium ions upon the transition temperature of MPA at high sodium chloride concentration (0.2 mole/l). The points are the observed temperatures and the lines represent the theoretical curves calculated from eq. (19), assuming the transitions temperatures for fully charged and neutral MPA to be 30.5°C and 48°C respectively at this ionic strength. Although correspondence between experiment and theory is in agreement with the value of K already taken, 0.5 $mole^{-1}$ l, the spread of transition temperatures implies (by way of eq. (19); the value of the integral is 1.72×10^{-2} C V m^{-2} for fully-charged lipid and zero for uncharged lipid) that the change in molecular

area at the transition is 12.3 Å^2, whereas the change observed by X-ray diffraction is only 6 Å^2 (K. Harlos and A. Blume, personal communication). The discrepancy, and the steepness of the experimental T_t/log [Ca^{2+}] curve, could be due to the fact that even at this high ionic strength the co-operativity observed in the binding of calcium at low ionic strengths is not entirely abolished. It is also possible that since the calcium binds to every *second* phosphate group, there exists an electrostatic attraction between the unneutralised groups, which are negative, and those bearing a calcium ion, which are on balance positive. This would tend to pull the head-groups together, thus raising the transition temperature of calcium-saturated MPA *. It would also represent a breakdown in the smeared-charge approximation made by the Gouy–Chapman theory, which cannot account for microscopic interactions within a surface once it is formally neutral. A similar raising of the T_t value of partly-protonated MPA has been attributed to the formation of intrafacial hydrogen bonds [19].

4.5.2. Behaviour at low salt concentration

The origin of the apparent co-operativity of calcium binding at low concentrations of 1 : 1 electrolyte is not clear. Although, as shown above (fig. 3), some of the lipid's head-groups are only slowly accessible to the calcium ions below the phase transition, it has already been observed [4] that prolonged sonication does not remove the splitting of the phase transition curve, so that it is unlikely to be an artefact due to inaccessibility of some of the head-groups. On no occasion have we observed the disappearance of the transition, as reported [23] for phosphatidic acid in the presence of excess calcium.

The separation into calcium-rich and calcium-depleted zones cannot be a simple consequence of the Gouy–Chapman theory, although some writers have claimed this; it has been shown in the references cited [1,14] that application of simple electrostatic theory always leads to a surface of homogeneous charge density, and that charge imbalance always leads to unfavourable energy changes.

When the molecular area of the lipid varies the situation becomes more complicated. It is possible to argue that the affinity of the calcium ions for the membrane in its ordered state (higher charge density) is greater than that for the membrane in its fluid state (lower charge density). Thus the onset of the phase transition of a partially-saturated MPA membrane would result in accumulation of calcium ions in those regions which were still ordered, reducing their electrostatic free energy and increasing their T_t (eq. (16)), while the depleted regions would have a lowered T_t. In consequence the passage from low to high temperature would involve the three steps [ordered membrane, uniform calcium distribution] $\xrightarrow{T_{t1}}$ [ordered, calcium-rich zones plus fluid, calcium-depleted zones] $\xrightarrow{T_{t2}}$ [fluid membrane, uniform calcium distribution]. T_{t1} is the lower and T_{t2} the higher observed transition temperature, and if the effect were strong enough these would correspond to the T_t values for almost fully charged and almost neutral MPA's respectively.

This effect would, however, be too small, as the following illustration shows. Let a membrane initially be uniformly half-saturated with calcium, so that σ equals $e/2f$. Let half of the membrane be in the ordered state ($f = f_A$) and half in the fluid state ($f = f_B$; $f_A < f_B$). The two surface densities of charge will therefore be different ($\sigma_A > \sigma_B$) and, in consequence, so will the surface potentials. Therefore a fraction δ of the calcium ions at the fluid surface will move to the ordered surface until the surface potentials are equalised (more exactly, until the chemical potentials of the calcium ions at the two surfaces are equal). If the membrane is in reasonably high concentration in the bulk solution — as it is, e.g., in an experiment designed to observe ion "pulses" caused by the membrane — then the total amount of calcium bound remains roughly constant and the surface charge densities at equilibrium of the ordered and fluid regions are given respectively by

$$\sigma_A = \frac{e}{2f_A}(1-\delta), \quad \sigma_B = \frac{e}{2f_B}(1+\delta). \tag{31}$$

Since the potentials at the two surfaces are equal it follows from eq. 4 or 26 that the surface densities σ_A and σ_B are also equal, whence from eq. (31)

$$\delta = (f_B - f_A)/(f_B + f_A). \tag{32}$$

This yields a value for δ of 0.07 ($f_A = 40$ Å^2, $f_B = 46$ Å^2), which, in view of the entropic opposition to non-zero values of δ, must be taken as an upper limit. The resulting predicted splitting of the transition temperature

* Such attraction has been observed in equimolar mixtures of anionic and cationic detergents in monolayers [17].

would be around one degree and would not be noticed. Furthermore, it would exist for all binding ions and at all salt concentrations *.

Thus if the splitting of the phase transition is due to the preference of calcium ions for the ordered membrane, the effect must be reinforced by some factor in addition to electrostatics. One such would be, for example, a chelating effect of adjacent phosphate groups which only came into play when the lipid was ordered, either because of the greater spacing between head-groups in the fluid lipid or because of their greater freedom of motion. But this would be reflected in a substantially greater K value below the transition temperature than above it, which should have been seen in the binding curves above; however, it was not.

Further possible origins of the phase separation induced by calcium and removed by salt are two-dimensional "precipitation" of calcium onto the head-groups, to form a lattice on the membrane surface, and possible face-to-face interaction involving separate bilayers. Such interpretations await structural investigation outside the scope of this study. It should be mentioned that the splitting seen with MPA is unlikely to be of biological importance, since most biological membranes are exposed to bulk 1 : 1 electrolyte concentrations well above 0.1 mole/l. However, there are several examples in biology of co-operative processes involving calcium binding [24], so that the phenomenon observed here may reappear in other, biological systems.

5. Summary

We may summarise our findings as follows:

(i) The degree of binding of calcium to MPA may be described empirically using the unmodified Gouy–Chapman theory to describe electrostatic interactions at the membrane surface, combined with an inherent binding constant of ca. 0.5 mole^{-1} l.

(ii) The Gouy–Chapman theory alone is unable to predict quantitatively the high value of the transition temperature of calcium-neutralised MPA or to predict even qualitatively the splitting of the phase transition curve, apparently due to phase separation, of MPA which is partly neutralised by calcium. Since this splitting is only observed at concentrations of 1 : 1 electrolyte well below 100 mmole/l, the Gouy–Chapman equations may be applied at normal physiological salt concentrations.

(iii) At these salt concentrations, the amount of calcium in the diffuse layer is small compared with the amount bound to the lipid surface. The surface potential can therefore be represented by eq. (26), and this equation may be combined with eq. (2) to find the amount of calcium bound, the surface charge density and the surface potential. For a cation of charge number Z, the general form of eq. (28), accurate at surface potentials above (kT/e) or 25 mV, is

$$\log K_{app} = \log K + Z(4.245 - \log C_{\pm} - 2\log f + 2\log \mu) \quad (33)$$

where f is the average molecular area (Å^2) for all lipids in the membrane and μ the fraction of these which carry a negative charge, the rest being neutral.

An approximate expression for the degree of dissociation is eq. (34a), expressed as f_t in eq. (34b). This is accurate to 0.03 in α if the calcium concentration is within two decades of that corresponding to the half-neutralisation point; it is shown as a broken line in fig. 2. If K is very small then to account for the screening calcium ions a lower limit for K_{app} of 0.02 should be used in eqs. (34).

$$\alpha = 0.52 + 0.17\,(\log K_{app} - \log [Ca_{free}])\,, \quad (34a)$$

$$f_t = 0.27 + 0.085\,(\log K_{app} - \log [Ca_{free}])\,. \quad (34b)$$

(iv) The apparent binding constant (i.e., the reciprocal of the free calcium concentration at the half-equivalence point) is given by a simple formula (eqs. (28), (33)), showing that an n-fold change in the concentration of free 1 : 1 electrolyte produces an (n^2)-fold change in the apparent binding constant for calcium, in contrast to the binding constant for protons, in which the change is only n-fold. Thus the balance of binding of these cations will be sensitive to small changes in the electrolyte medium surrounding the membrane. This effect could be still more important for multivalent cations (proteins) bound electrostatically to the membrane.

(v) The effect of the fraction of charged groups in the membrane is even greater. Thus in the case $C_{\pm}$ = 0.15 mole l^{-1}, f = 50 Å^2, μ = 0.2, the enhancement of

* It would also vanish at very low lipid concentrations, since then plenty of calcium would be available in solution. We tested this at lipid concentrations down to 2.5 × 10^{-5} mole/l: the splitting persisted.

calcium ion binding (K_{app}/K_0) is a factor of only 3.5; however at $\mu = 0.5$ this factor rises to 137 and at $C_{\pm} = 0.1$ mole l^{-1} to 309. Thus the membrane's affinity for calcium varies widely within the range of conditions available to a biological membrane.

(vi) "Steps" or "pulses" in calcium concentration may be produced by the ordered-fluid phase transition, just as observed in the case of protons. The magnitude of such steps already reported was shown to be in accord with the theory. However, as has already been shown [21] for protons, the magnitude of such steps is small (here a factor of ca. 2), so changes in enzyme activity induced by a change in the state of a membrane and mediated by calcium ions are unlikely to be great enough to enable the membrane to act as an on/off switch for an enzyme unless enhanced by some additional co-operative effect.

(vii) We are in agreement with the conclusions of Eisenmann et al. [5] that the mechanism of the reduction of membrane surface potential by divalent ions can occur to a large extent through screening rather than binding. However the total amount of calcium taken into the double layer is under many circumstances not strongly dependent upon K (cf. fig. 8). A distinction between bound and free calcium in the double layer may be necessary for an understanding of the relationship between the composition and the function of a mambrane. For example, if the physical properties of a membrane are to be modified by the attachment of calcium, the membrane must have a high K value; in activating an enzyme which requires calcium, only the free ions are effective, so a low K value, which boosts the surface potential, may be more appropriate.

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