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The determination of binding site density and association constants for monovalent cation adsorption onto liposomes made from mixtures of zwitterionic and charged lipids

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The Gouy-Chapman-Stern theory predicts that the shape (but not the magnitude) of the surface potential dependence on the electrolyte concentration mainly reflects the screening of the surface charge but not cation adsorption. So this dependence does not allow to determine two parameters of the theory (the surface density of binding sites (S) and the association constant (K)) simultaneously. Therefore the fitting procedure for the determination of S and K was suggested as a test of cation adsorption for the surface or zeta potential measurements at a fixed electrolyte concentration but with a variable ratio of charged and neutral components in the lipid mixture. This procedure was applied to the electrophoretic measurements made by the method of photon correlation spectroscopy in the suspensions of PS/PC or CL/PC liposomes in the monovalent electrolytes. For KCl and NaCl electrolytes it only leads to the different association constants (about 0.2 and 0.8 M^{-1}, respectively) corresponding with data from the literature but to the same value of the surface charge density (about -16 $\mu C/cm^2$) which is smaller than usually postulated for PS membranes. The fitting of zeta potentials measured in tetraalkylammonium salts shows a small cation adsorption but a large surface charge density (about -21 $\mu C/cm^2$).

Introduction

Most of the information about the binding of ions to biological membranes is obtained from experiments with lipid membranes of different compositions. There are two main questions of biological interest to be answered by these experiments: (1) what is the physical nature of adsorption of ions and (2) how is it connected with the structure of the membrane. The popular theoretical basis of this investigation is the theory of diffuse electric layer combined with the Langmuir adsorption isotherm – the Gouy-Chapman-Stern theory (GCS) [1–3]. It has at least two parameters for the stoichiometry and energetics of adsorption for each kind of ions: the surface density of binding sites S and the association constant K. The association constants for many inorganic cations are obtained from the measurements of the electrophoretic mobility of liposomes [4–6]. It is shown that cations are adsorbed preferentially on negatively charged lipids which are assumed to be only binding sites for monovalent cations. The surface density of binding sites was assumed to have the same fixed value at any experimental conditions and there are no attempts to test this concept experimentally by electrophoresis.

The surface density of binding sites S is determined mostly by the surface area per lipid molecules (w) which is usually obtained from the measurements using crystal samples, monolayers or multilayers of lipids. There is no confidence that the state of ionization and packing of lipid molecules in these systems are identical to those in bilayer lipid membranes. There is some evidence that the phase transition temperature of lipids depends on the type and concentration of the electrolyte [7,8], that the electrophoretic mobility of liposomes changes at the phase transition temperature of lipids [7,9]. Moreover, the surface pressure in PS monolayers is higher in tetraalkylammonium electrolytes than in the case of inorganic cations as a possible result of different

Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; CL, cardiolipin; TMA, tetramethylammonium chloride.

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electrostatic interactions between lipids [10]. This means that the surface charge density may depend on experimental conditions not only due to negative charge neutralization by cation adsorption. Thus to test the simpliest theory of cation adsorption at the lipid membranes one must determine the values of both parameters of the GCS theory from the same experiment simultaneously. If there is no 'a priori' information about the number of charge groups per lipid molecule or about their state of ionization, electrophoretic measurements will become the only way to obtain that information by experimental determination of S [11,12].

The correct test of the hypothesis on cation adsorption presumed some fitting procedure for the determination of S and K values which are to be compared with those in the case of an indifferent electrolyte. As it follows from the GCS theory the shape (but not the magnitude) of the surface potential dependence on the electrolyte concentration mostly reflects the screening effect but not the adsorption of cations. So the experimental data on this dependence with the usual precision of the electrophoretic measurements do not allow to find the values of both parameters simultaneously. In order to do this we used the fitting procedure applied to the measurements of surface or zeta potentials at fixed electrolyte concentration by varying the content of the charged component in the lipid mixture. The measurements were performed with PS/PC and CL/PC liposomes in sodium, potassium and tetraalkylammonium chlorides. The GCS theory parameters obtained do not depend on the electrolyte concentration, and the association constants are in accordance with literature data.

Theory

The electrokinetic (zeta) potential ζ is measured at the hydrodynamic plane of shear located at the distance $x = \delta$ from the charged surface. In the case of 1:1 electrolyte the potential dependence on the distance x is described by the theory of the diffuse electric layer [1,2,13]:

$$\tanh(e\phi(x)/4kT) = \tanh(e\phi(0)/4kT)\exp(-\varkappa x) \tag{1}$$

where $\varkappa^{-1} = (2e^2c_0/\varepsilon\varepsilon_0kT)^{-1/2}$ is the Debye length, ε is the relative dielectric permeability of the medium, ε_0 is the electric constant, c_0 is the bulk electrolyte concentration and e is the elementary charge. The surface potential $\phi(0)$ may be obtained by Formula 1 with a given δ and measured $\zeta = \phi(\delta)$. Then the cation concentration adjacent to the charged surface $c(0)$ and the surface charge density σ may be calculated by the Boltzmann relation

$$c(0) = c_0\exp[-ze\phi(0)/kT] \tag{2}$$

and the Gouy-Chapman equation

$$\sigma = (8c_0\varepsilon\varepsilon_0kT)^{1/2}\sinh[ze\phi(0)/2kT], \tag{3}$$

where z is the charge number of ions. The cation adsorption is described by the Langmuir adsorption isotherm with σ and S being the free and total surface sites density, respectively, and K being the association constant:

$$\sigma/S = (1 + Kc(0))^{-1} \tag{4}$$

In the simplest model for adsorption of monovalent cations S is proportional to the content of the single charged lipid molecules in the lipid mixture and the maximal value $S_{max} = Q$ corresponds to 100% of charged lipids.

As follows from Eqns. 1–4 the magnitude of the surface potential depends on cation adsorption but the shape of its dependence on the electrolyte concentration mainly reveals the screening effect. This shape changes a little in the case of cation adsorption but it is not sufficient to distinguish the experimental curves from the case of an indifferent electrolyte. To show this let us consider the case of large potentials $\phi(0) > kT/e$. The Eqn. 3 has an asymptotic form:

$$\sigma \approx \sigma^* = A(c_0\exp[-ze\phi(0)/kT])^{1/2} \tag{5}$$

where $A = (2\varepsilon\varepsilon_0kT)^{1/2}$. Eqns. 2, 4 and 5 determine the characteristic charge σ^* by the parameters of adsorption S and K:

$$S = \sigma^*[1 + K(\sigma^*/A)^2] \tag{6}$$

The value σ^* is equal to the total surface density of binding sites S only in the case of an indifferent electrolyte ($K = 0$). In general, σ^* is the surface density of free binding sites at small electrolyte concentration when the surface potential has a linear dependence on $\log(c_0)$. This line crosses the abscissa at the point $c^* = (\sigma^*/A)^2$.

As it follows from this simple analysis the effect of cation adsorption on the surface charge is not negligible even at infinitely low electrolyte concentration. This result is known as the 'contact value theorem' (see Ref. 3, and references cited therein). The cation concentration adjacent to the surface has an asymptotic value c^*, independent on the bulk electrolyte concentration. It means that the density of free binding sites becomes constant but not equal to the total density of binding sites: for instance the surface charge density σ^* does not exceed $S/2$ for $K = 1\ M^{-1}$ and $S = -10\ \mu C/cm^2$.

In the range of low electrolyte concentration, where $\phi(0) > kT/e$, one can obtain no more than the value of σ^* from the experiment. As it follows from Eqn. 6 this value does not allow to determine the two parameters of

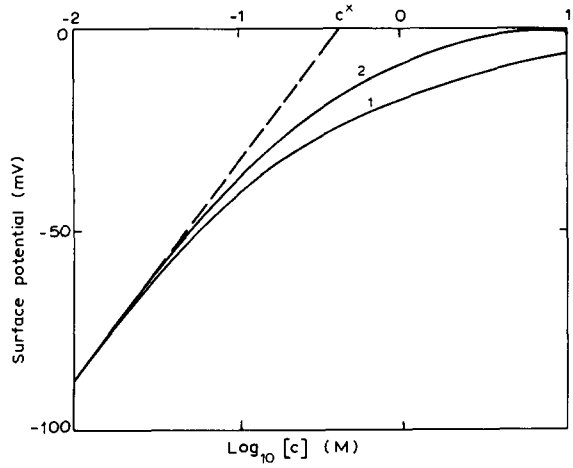

Fig. 1. Surface potential calculated at different concentrations of an indifferent electrolyte (curve 1) and that in the case of cation adsorption ($K = 1.5$ M^{-1}, $S = -16$ μC/cm^2 (curve 2) under condition of the same asymptotic value for the surface charge density $\sigma^* = -3$ μC/cm^2. The parameter c^* is the asymptotic value of the cation concentration adjacent to the surface.

the theory independently. In order to test the cation adsorption it is, therefore, necessary to use the experimental data at a moderate concentration where the behaviour of an indifferent electrolyte is quite different from the case of cation adsorption. We depicted this range of concentration in Fig. 1 and Fig. 2 by the curves calculated using Eqns. 1–4 (with $\delta = 0$) and assuming that at low concentration of both electrolytes they have a similar dependence $\phi(c_0)$ and equal σ^* value. At high electrolyte concentration the surface potential

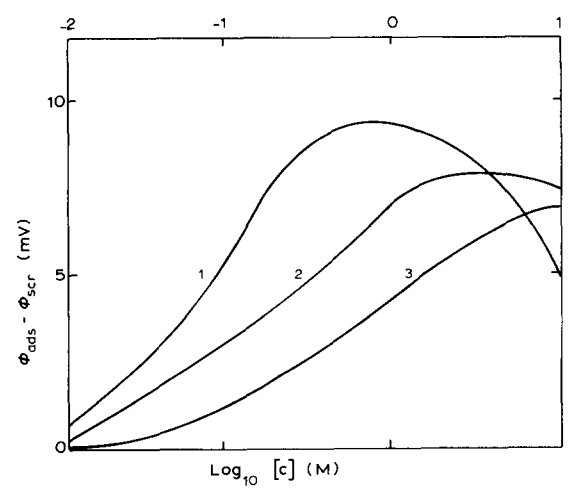

Fig. 2. Difference $\Delta\phi = \phi_{ads} - \phi_{scr}$ of the surface potentials calculated at different electrolyte concentrations in the case of cation adsorption (ϕ_{ads}) and in the case of the screening effect only (ϕ_{scr}) for an indifferent electrolyte. The surface density of the binding sites is assumed to be $S = -16$ μC/cm^2 for each curve and the association constants K are calculated by Eqn. 6 under the condition that the asymptotic value of the surface charge density σ^* is the same for both kinds of electrolyte. The values of K (M^{-1}) and σ^* (μC/cm^2) are 10 and -3.5 (curve 1), 1.0 and -6.8 (curve 2), 0.2 and -10.0 (curve 3), respectively.

changes because of the screening effect or by decreasing the surface charge due to cation adsorption, too (Fig. 1). The difference between the curves depends on the parameters of adsorption: it has a maximal value at the electrolyte concentration about 1 M and does not exceed 10 mV (Fig. 2). High precision of the surface potential measurements is necessary within this range of electrolyte concentration as to answer the question whether the electrolyte is an indifferent or not. In the case of zeta potential measurements (when $\delta > 0$ and the difference between the curves becomes smaller) this problem is more difficult.

This problem may be easily solved in an experiment with a change of the surface charge by combination of neutral and charged components at the fixed electrolyte concentration. If the neutral (zwitterionic) lipids do not adsorb a cations they can only decrease the surface density of binding sites in the outer layer of liposomes and the effective association constant will not depend on the lipid composition. This condition was used here for the fitting procedure: the best value of the surface density of binding sites is the value which makes K independent of the percentage of charged lipids in the mixture.

The association constant K may be found from Eqn. 4 for liposomes with different $\alpha = S/Q$ (i.e., the relative part of their surface area occupied by the charged component):

$$K = ((\alpha Q/\sigma) - 1)/c(0) \tag{7}$$

The maximal surface density of binding sites Q is the highest charge density of the liposomes of charged lipids ($\alpha = 1$).

If one has got the Q value it will be possible to obtain the best K value by averaging K in Eqn. 7 over the experimental points. Otherwise both parameters must be found from the experiment. The optimal Q value is that value which gives the K values independent of α, i.e., the dependence of K on α must be a line parallel to the α axis. The best linear approximation of the experimental points $K(\alpha)$ gives the equation

$$K(\alpha) = L\,\alpha + M \tag{8}$$

where the angular coefficient L and the constant M depend on the value of Q. Optimal Q and $K = M$ value correspond to the condition $L = 0$ and give the best fitting of the experimental data.

Materials and Methods

Bovine brain phosphatidylserine was prepared in the laboratory of Lipid Chemistry of Vladivostok State University (U.S.S.R.); egg phosphatidylcholine was prepared in the laboratory of Membrane Biochemistry of the Institute of Bioorganic Chemistry of the Academy

of Sciences, U.S.S.R. Cardiolipin was obtained from Kharkov Factory of Bacterial Preparations (U.S.S.R.). The purity of the lipid samples was controlled: the samples gave a single spot by one-dimensional TLC (chloroform/methanol/water, 65 : 25 : 4, v/v). Multilamellar liposomes were prepared by evaporation of a chloroform solution of the lipid mixtures and by gentle shaking in the electrolyte. The final lipid concentration in an aqueous sample was 1 mg/ml.

Aqueous solutions of alkali metal chlorides 'ultrapure' from Reachim (U.S.S.R.) and buffers (Calbiochem) were made in bidistilled or monodistilled water in the presence of EDTA (0.025–0.5 mM). Tetramethylammonium chloride 'pure' from Reachim (U.S.S.R.) and choline chloride (Calbiochem) were recrystallized from methanol/acetone or acetone solution, respectively.

The electrophoretic mobility μ of liposomes was measured by the commercial device 'Zetasizer-2' (Malvern, U.K.) based on the method of photon correlation spectroscopy [14,15]. The method uses the autocorrelation function of the light scattered in colloid solution measured by a photon counting system. The method of liposome preparation used here does not give a narrow size distribution of the particles: the $1/z$-average diameter of the liposomes is about several μm with a polydispersity larger than 0.6. The particles move in an electric field of known strength E in the interference pattern of two laser beams. The main frequency of scattered light fluctuations (due to the Doppler effect) ν, depends on the particle velocity v and the fringe spacing l (0.488 μm in our case). They were obtained by the Fourier transform of the autocorrelation function and connected with the electrophoretic mobility μ. Then zeta potential ζ may be obtained using the Smoluchovsky relation [13–15]:

$$\nu = v/l = \mu E/l;\ \mu = \varepsilon\varepsilon_0\zeta/\eta \qquad (9)$$

where η is the viscosity. Each measurement gives a spectrum of liposome mobility which sometimes had a number of peaks, and its width is more sensitive to the quality of the lipid preparation than TLC. Here we used liposome suspensions with a single peak spectrum averaged in the range of Doppler frequencies up to 1 kHz.

The measurements of the electrophoretic mobility μ were performed at the fixed electrolyte concentrations at 22° C with liposomes of different mixtures of neutral (PC) and charged (PS, CL) lipids. If the content of lipids in the outer layer of the liposomes is equal to the initial mixture in chloroform solution the surface area occupied by the charged component can be easily calculated by

$$\alpha = rR/(rR+1);\ r = V_1/V_2;\ R = (w_1/m_1)/(w_2/m_2) \qquad (10)$$

V_i, w_i and m_i correspond to sample weight, surface area and molecular weight of the charged ($i = 1$) and neutral ($i = 2$) components of the lipid mixture, respectively. The data shown in Table I were obtained with $R = 1$ for PS/PC and with $R = 1.297$ for CL/PC liposomes: $w_{PC} = 60$ Å^2, $m_{PC} = 750$ and $w_{CL} = 146$ Å^2, $m_{CL} = 1407$ [16].

Results

The electrophoretic mobility μ of liposomes of different composition (7–10 values of α) was measured at three concentrations of each kind of electrolyte (10, 50 and 100 mM). The experimental data on zeta potentials of PS/PC liposomes in KCl solutions are shown in Fig. 3. Theoretical curves were calculated with optimal values of the maximal surface charge density Q and the association constant K which were obtained for each series of measurements at the fixed electrolyte concentration. The experimental data and theoretical curves for the other electrolytes with the same concentration are shown in Fig. 4. Data on similar measurements in other electrolytes are not presented here, but the data analysis was qualitatively the same.

The procedure of the determination of optimal values for Q and K is depicted in Fig. 5 and Fig. 6 with the experimental data taken from the curve 3 in Fig. 3. For each value of α and the measured zeta potential the surface potential $\phi(0)$ was calculated by Eqn. 1 and the values of cation concentration adjacent to surface $c(0)$ and charge density σ were calculated by Eqns. 2 and 3. The location of the shear plane was chosen 0.2 nm from the surface [3,5,17]. The experimental values of the zeta potential $\zeta(\alpha)$ measured at the fixed electrolyte concentration give a set of association constants $K(\alpha)$

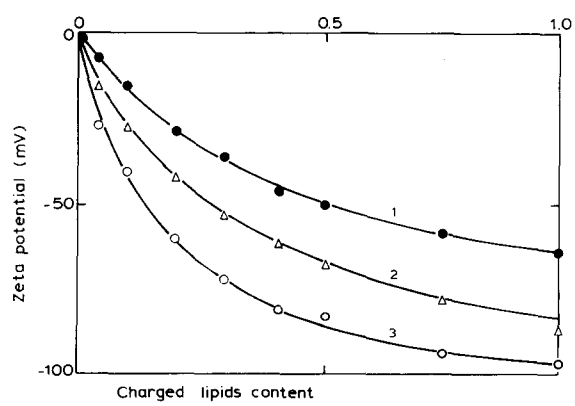

Fig. 3. Zeta potentials of PS/PC liposomes measured for different PS content in the lipid mixture. The electrolyte compositions are: 100 mM KCl, 5 mM Tris (pH = 7.4), 0.5 mM EDTA (●); 50 mM KCl, 2 mM Tris (pH = 7.3), 0.5 mM EDTA (△) and 10 mM KCl, 2 mM Tris (pH = 7.2), 0.5 mM EDTA (○). Theoretical curves are calculated for the optimal values of the maximal surface density of binding sites Q (μC/cm^2) and the association constant K (M^{-1}): −14.5 and 0.16; −15.5 and 0.19; −14.6 and 2.0 for curves 1–3, respectively.

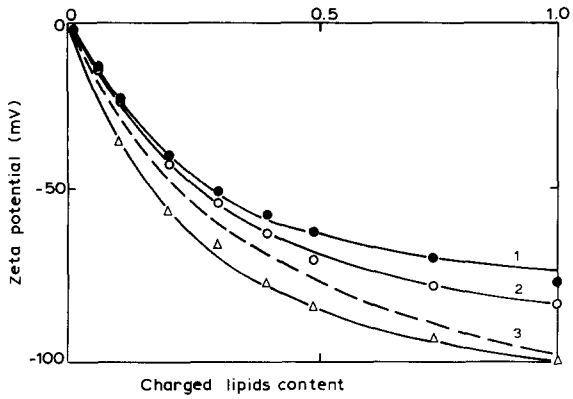

Fig. 4. Zeta potentials of PS/PC liposomes measured for different PS content in the lipid mixture at the same concentration (50 mM) of different electrolytes: NaCl (●), KCl (○) and TMA-Cl (△). Theoretical curves were calculated using the optimal values of the adsorption parameters K (M^{-1}) and Q ($\mu C/cm^2$): 0.53 and −15.9; 0.19 and −15.5; 0.05 and −21.7 for curves 1–3, respectively. The dashed curve is for $K = 0$ and $Q = -16\ \mu C/cm^2$.

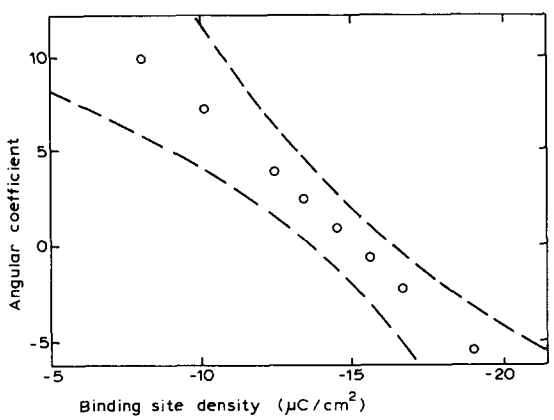

Fig. 5. Angular coefficient L of the line approximation Eqn. 8 calculated for different values of the maximal surface density of binding sites Q using the experimental data of curve 3 in Fig. 3. The dashed lines show the standard errors.

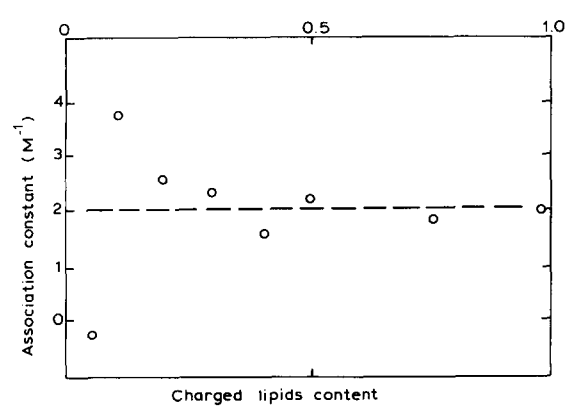

Fig. 6. Association constants K calculated according to Eqn. 7 for the experimental points of curve 3 in Fig. 3 under the condition $L = 0$. Optimal values of the adsorption parameters (and standard errors) are: Q ($\mu C/cm^2$) = -14.6 ± 0.9 and $K(M^{-1}) = 2.0 \pm 0.7$.

TABLE I

Optimal values of the maximal surface density of binding sites Q and of the association constant K obtained by fitting the experimental data with linear approximation (Eqn. 8) under the condition $L = 0$

Our measurements were made in the presence of EDTA at 0.5 mM, 0.3 mM [a] or 0.1 mM [b].

Concentration of electrolyte (mM)		Association constant, $K \pm$S.E. (M^{-1})	Binding site density, $Q \pm$S.E. ($\mu C/cm^2$)	Source (Ref.)
Phosphatidylserine				
KCl	10	2.0 ±0.7	−14.6±0.9	
	50	0.19±0.1	−15.5±0.5	
	100	0.16±0.05	−14.5±0.3	
NaCl	10	1.1	−17.6	6
	10	0.81	−16.3	18
	50 [a]	0.53±0.2	−15.9±0.7	
	100 [b]	0.74±0.4	−16.0±2.4	
	100	0.79	−22.2	18
	3.1	0.78	−23.9	5
	15	0.87	−26.6	5
	114	1.3	−35.9	5
TMA	50	0.05±0.1	−21.7±1.0	
	100	0.10±0.4	−20.0±3.5	
Choline	50	0.11±0.2	−19.5±1.2	
TMA	3.1	0.27	−28.6	5
	12	0.12	−30.7	5
	100	0.13	−46.3	5
Cardiolipin				
NaCl	10	3.3 ±0.6	−15.1±0.8	

corresponding to arbitrary Q value. The angular coefficient L of the linear regression (8) for $K(\alpha)$ was calculated at different values of Q and it showed a linear dependence on Q (Fig. 5). This procedure allows us to determine the intersection point $L = 0$ in all experiments by linear extrapolation of $L(Q)$. The optimal values of Q and K correspond to the condition $L = 0$ and to a minimal standard deviation of the experimental points from the theoretical curves which did not exceed 2–3 mV. There was no systematic deviation of points $K(\alpha)$ or $\zeta(\alpha)$ around the line with $L = 0$.

Standard errors of optimal Q and K values increase at low electrolyte concentration. As a result of adding EDTA, the mobility spectra became narrower, zeta potentials increased, and the K values and the standard errors of Q and K are decreased. These effects seem to be due to multivalent cation contaminations. So we made most of the measurements in the presence of EDTA. In Table I we collected the results of the data analysis obtained in this work at high concentrations of different electrolytes and of those data obtained by other authors. In the latter case errors are not shown: the experimental data on zeta potentials are taken from the figures of the papers quoted.

Discussion

Our experimental data on liposome electrophoretic mobility obtained by the light correlation spectroscopy method are in a good agreement with the known data of the traditional method of microelectrophoresis. Our maximal value of the zeta potential of liposomes made from phosphatidylserine are smaller (no more than 5–10 mV) than those under the same conditions in Ref. 5. We attribute this difference to the mobility averaging procedure which is quite different for both methods. Other reasons may be connected with possible admixtures of neutral lipids in PS preparations and multivalent cations in water solutions. We assume the most reliable values of K to be found at high electrolyte concentrations and in the presence of EDTA. They are close to the literature values 0.5–0.7 M^{-1} for Na (0.6–1.0 M^{-1} in Refs. 5, 6 and 18) and 0.16–0.19 M^{-1} for K (0.2 M^{-1} in Ref. 5). Association constants obtained for TMA and choline cations are small but not negligible, i.e. both electrolytes do not behave as an indifferent ones.

The conclusion about TMA as an indifferent electrolyte was drown in Ref. 5 by a comparison of zeta potentials measured at three TMA-Cl concentrations with the theoretical curves for $K = 0$ and $Q = -23$ $\mu C/cm^2$. The agreement is not very good: there is a systematic deviation of the theoretical curves from the experimental points which is not explained by the authors. They also mentioned the inhomogeneity of the liposome mobility which was assumed to be due to a relaxation effect. So the measurements of the zeta potential in Ref. 5 were made for the largest liposomes (> 10 μm) with highest mobility. It makes the zeta potential values overstated, and unrealistic high values of Q in our treatment of the data in Ref. 5 are the result of it. Our data are in better agreement with the data of the same authors in their following paper [6].

The presence of all kinds of admixtures of neutral lipids in phosphatidylserine is essential for the determination of the association constant K but it does not affect the optimal value of the maximal surface density of binding sites Q. The same relative alterations of each α value in Eqn. 10 can only lead to a new scale of the α axis (see Fig. 6) and do not affect the condition $L = 0$ in Eqn. 8. Moreover, we made a fitting procedure with the same experimental data with different assumptions about the location of the shear plane, δ. Variation of δ in the range 0.1–0.5 nm leads to a variation in association constant K values of about two orders of magnitude. Over the same range of δ the optimal value of the binding site density Q changes only by a few percents. So one can more reliably find the last parameter from zeta potential measurements than the first one.

The theory used here is suitable in the case of electrolyte–surface interaction mediated by cation adsorption only. This adsorption was assumed to be preferential to negatively charged binding sites, and it was assumed that there are no notable changes of surface charge due to ion binding to PC and to phosphate groups on PS and CL. This simplification may be incorrect in general ([9,12]), but if the values of the association constants of cations and anions are very similar the effect of their binding on the zeta potential may be negligible. Our experiments supported this assumption by two facts: zeta potentials of PC liposomes ($\alpha = 0$) are about zero for each electrolyte solution and the optimal values for K and Q do not depend on its concentration.

The surface density of the binding sites Q for sodium and potassium chlorides shows practically the same value. This means that the difference between the zeta potentials measured at the same concentration of both electrolytes reveals only the different quantity of adsorbed cations, as was assumed in Ref. 5. For TMA and choline chloride solutions we have got another result. The qualitative difference of electrolytes with inorganic and tetraalkylammonium cations is shown in Fig. 3. The dotted theoretical curve was calculated with $K = 0$ and $Q = -16$ $\mu C/cm^2$. The latter value is about the same as that obtained for sodium and potassium chlorides. So with a fixed Q value at $\alpha = 1$ one can obtain the values of the association constants close to those in Ref. 5, i.e., TMA-Cl looks like an indifferent electrolyte. The experimental points at small α lead to high Q and K values in the case of tetraalkylammonium chlorides.

The simplest theory of cation adsorption used here reveals a difference between organic and inorganic cations but it does not explain it. One can suppose some enhancement of anion binding to lipids or a larger distance between the charged surface and the shear plane ($\delta \approx 0.3$ nm) instead of cation adsorption in the case of tetramethylammonium chlorides. But it is not enough to describe the shape of the experimental curves for a small α. It is also necessary to assume a different influence of both electrolytes on lipid packing or their state of ionization. A higher Q value in the case of TMA-Cl in comparison with alkali metal chlorides can lead to a stronger electrostatic repulsion of lipids. This conclusion is supported by the observation of the effect of these electrolytes on the surface pressure in lipid monolayers and liposomes [10].

In principle, the lipid composition in chloroform solutions and in the outer monolayers of liposomes may be different. The equilibrium of lipid molecules between liposomes and aqueous solution depends on the critical concentration of micelle formation and on the lipid structure. It may affect the composition of the outer layer especially at low electrolyte concentration [19]. Moreover, intermolecular interactions can change the surface area per molecule in the lipid mixture [20]. Both factors should lead to a systematic deviation of the points from a linear dependence $K(\alpha)$ which was not observed in our experiments. As it follows from Eqn. 5

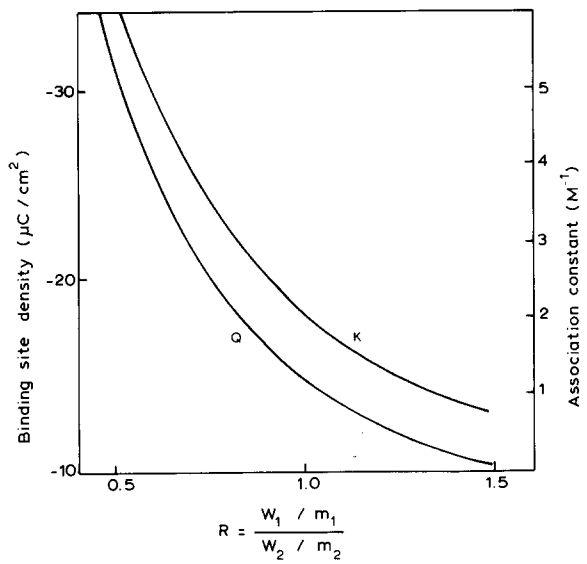

Fig. 7. Maximal surface density of binding sites Q and association constant K obtained by fitting the experimental data presented by curve 3 in Fig. 3 for different values of the parameter R in Eqn. 10 (the ratio of surface areas w_i and molecular weights m_i for charged ($i = 1$) and neutral ($i = 2$) components in the lipid mixture).

in the case of the linear dependence of the surface charge on PS content in the lipid mixture one should obtain a log-shape of the surface or zeta potential dependence on the PS percentage. Exactly the same observation was made in Ref. 21 by means of the plate electrophoresis technique with small PS/PC liposomes (< 0.2 μm). It supports the validity of the assumption on the lipid mixture used in Eqn. 10.

A more important factor for data analysis is the determination of the real value of α, i.e., the real ratio of the surface areas of neutral and charged components in the lipid mixture. For instance, the value of w_{PS} is in the range of 43 to 68 Å^2 for different structures of hydrophobic tails and ionic compositions in water solution [22]. The method used here for Q and K determination needs information about the ratio of the surface areas per lipid for both kinds of molecules (parameter R in Eqn. 10), but no information about their absolute values. Unfortunately, we had no such information about our lipid samples. So we studied the dependence of our results on the relation R using the experimental data of curve 3 in Fig. 3. The optimal values of Q and K obtained from these data with different values R are depicted in Fig. 7. The value of $Q = -23$ μC/cm^2 which is widely used in literature corresponds to value for R of about 0.6. Our results (see Table I) gives the values of the maximal surface density of binding sites Q smaller than those in the papers quoted. One possible explanation of this discrepancy is an incorrect value of R which we used in our calculations. Another one is the existence of the intermolecular interactions in the lipid layers which can decrease the ionization of the lipid molecules. For instance, surface areas per lipid molecule measured for monolayers at the air/water interface under a surface pressure of 30 dyn/cm^2 are about $w_{PS} = 50$ Å^2 [23] and $w_{PC} = 65$ Å^2 [20] and this corresponds to $R = 0.77$. Moreover, a decrease of the surface charge density is possible in the case of acid lipids due to intermolecular hydrogen binding [24].

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