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IONIC STRUCTURE OF PHOSPHOLIPID MEMBRANES, AND BINDING OF CALCIUM IONS*

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SUMMARY

Radiotracer measurements of adsorption showed that the binding of Ca^{2+} with the phosphate groups of phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine monolayers depended largely on the molecular conformation of the lipid in the monolayer. The monolayers of phosphatidylethanolamine and phosphatidylcholine bind small amounts of Ca^{2+} when the lipid molecules are packed closer than 150 $Å^2$ per molecule. The distribution of bound Ca^{2+} on both phosphate and carboxyl groups of phosphatidylserine was analyzed from the differences in accessibility of Ca^{2+} to each group. The amount of Ca^{2+} binding is larger for the carboxyl than the phosphate groups of phosphatidylserine by a factor of about 3 at molecular areas larger than 150 $Å^2$/molecule at pH 7.5 and Ca^{2+} 0.05 mM. The apparent reactivities of these phospholipids to Ca^{2+} varied in the order phosphatidylserine > phosphatidylethanolamine $\gg$ phosphatidylcholine. The low reactivity of phosphate groups of phosphatidylethanolamine and phosphatidylcholine may reasonably be understood as a lowered ionic nature of the phospholipid owing to the intra- or intermolecular neutralization of the charge between ammonium cation and phosphate anion groups of the lipids. The Ca^{2+} binding is explained by taking into account those ionic equilibria of the acid and base dissociations, the intra- or intermolecular neutralization among phosphate, carboxyl and ammonium groups, and the calcium soap formation at each anionic site. The equilibrium constants, which determine the ionic properties of these phospholipids, are calculated from the adsorption data of Ca^{2+} at various pH values, and at various degrees of molecular packing of the phospholipid monolayers.

INTRODUCTION

After Gorter and Grendel[1] successfully showed a bimolecular structure of phospholipid for erythrocyte membrane from their extraction measurements, Danielli

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and Davson[2–4] confirmed and improved the model, and now, the leaflet bilayer structure of phospholipids is still generally recognized as the principal structure of the biological membrane.

A number of studies on the physicochemical properties of reconstituted membrane of phospholipid has hitherto been presented in order to simulate the natural biological membrane and to elucidate the molecular mechanisms of various physiological functions of the biomembrane, such as nervous excitation, active transport, muscular contraction and so on.

Three types of reconstituted membranes were generally involved in these studies. They are the black lipid bilayer[5], the smectic mesophase of liquid crystalline phospholipids[6], and the monomolecular layer of phospholipids.

The monolayer properties, although standing only for the one side of the membrane, are still thought to give more basic and physically meaningful information which might be helpful to understand various membrane phenomena at the molecular level.

Meanwhile, the roles of Ca^{2+} in various biomembrane phenomena as mentioned above, were found to be important by many investigators[7–11]. Kimizuka and Koketsu[12], Rojas and Tobias[13,14], Hauser and Dawson[15], and other workers have presented extensive work on the binding of Ca^{2+} to the monolayers[16] or vesicles [17–20], as well as on the competition of the Ca^{2+} with alkali metal ions or various organic compounds which have physiological implications[14,15,21]. The effect of Ca^{2+} on the physicochemical properties of phospholipid monolayers[22–25] or bilayers[26,27] has also been studied by many investigators.

In spite of the numerous work hitherto presented, those experimental data are sometimes hard to correlate with each other by a unified treatment based on the ionic structure of phospholipid membrane, probably because of the complex ampholytic nature of most phospholipids.

In this report, some trials were made to explain our experimental results, together with those obtained by other investigators, for the binding of Ca^{2+} with phospholipids by taking into account several ionic equilibria among the functional groups in phospholipid monolayers and those ions in the hypophase.

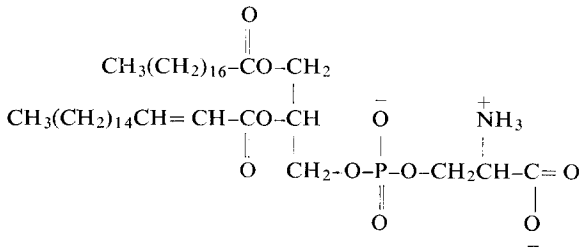
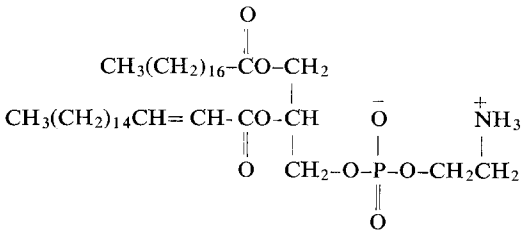
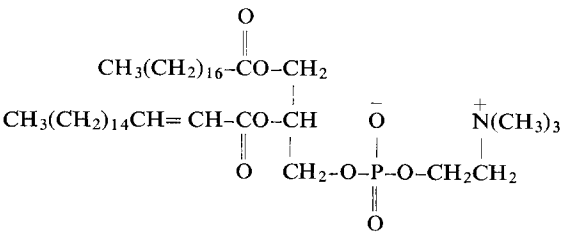
MATERIALS AND PROCEDURE

The phospholipids used are three of the major constituents of the biological cellular membrane. They are phosphatidylserine which was extracted from bovine brain, phosphatidylethanolamine and phosphatidylcholine extracted from egg yolk. Stearic acid was also used for comparison. The chemical formulae are shown in Table I, in which the alkyl chains are assumed to have one oleic and one stearic group.

Part of the phosphatidylserine used, was kindly given by Dr D. Papahadjopoulos of Roswell Park Memorial Institute of Buffalo, which was extracted from bovine brain and refined to a purity of more than 99% for phosphatidylserine and 86% for the alkyl chain of 18 carbon atoms. Phosphatidylethanolamine, phosphatidylcholine and the rest of the phosphatidylserine were bought from Supelco (Bellefonte, Pa.) and Applied Science Laboratory (State College, Pa.), each said to have the purity of 96 + %. The molecular weights were calculated from the assumption that the fatty acid chains of these phospholipids are one stearic and one oleic chain. This was

TABLE I

PHOSPHOLIPIDS

Compound	*Formula*
Phosphatidyl-L-serine	
Phosphatidylethanolamine	
Phosphatidylcholine	

confirmed by Dr Papahadjopoulos[28], as being the main constituent for phosphatidylserine. All phospholipids were sealed in glass ampoules with nitrogen gas, and stored in a refrigerator until needed. Stearic acid used was obtained from the Applied Science Laboratory and has single peak purity by gas chromatography. The benzene (Fischer, Scientific grade) used as a spreading solvent was purified over silica gel, and left no measurable surface pressure increase after evaporation when spread on the aqueous surface, even at the high compression caused by reducing the surface area to 1/10 of the original. The water used was distilled three times, including the process of alkaline permanganate treatment. The surface tension of the distilled water showed no measurable change after prolonged aging under the condition of the present experiment, and compared well with that of fresh water, within the ± 0.3 dyne/cm error of measurement. This indicates that there was no organic contamination of the surface during the whole experimental procedure. The pH was controlled with Tris–HCl (certified reagents of Fischer Scientific Company, Fair Lawn, N. J.) buffer solution. All experiments were carried out at room temperature

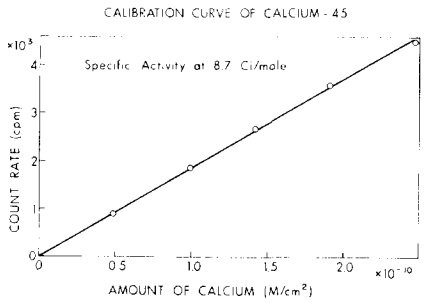
(25±1 °C), and in a nitrogen atmosphere to avoid both the oxidation of the phospholipid and the unfavorable effect of CO_2 on the surface pH and on the adsorption of solute[29]. Evaporation of water was also prevented by passing the nitrogen gas through a humidifier, and this eliminated every possibility of a change of surface concentration by evaporation[30]. The surface pressure *vs* molecular area relations of these phospholipids and stearic acid showed good agreement with those curves obtained by other investigators[31] and confirm indirectly that the purities of the lipids were high.

The radioactive ^{45}Ca was obtained as the chloride in an aqueous solution of HCl from International Chemical and Nuclear Corporation, Irvine, Calif. An aqueous solution of non-radioactive $CaCl_2$ (certified reagent of Fischer Scientific Company, N.J.) was used to control the specific activity of radioactive calcium. The concentration of Ca^{2+} was determined by EDTA titration using murexide as the indicator. The specific activity of ^{45}Ca was varied from 2.6 to 500 Ci/mole according to the concentration of Ca^{2+} in the substrate solution in order to give the optimal accuracy for the adsorption measurement. The principle of the adsorption measurement adopted here is the standard procedure[32,33] utilizing the radioisotope which emits the soft β ray, originally devised by Dixon and Salley[34], Annianson[35], and somewhat modified by the author[36].

The benzene solutions of stearic acid, phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine were spread on the clean aqueous surface of 6.15 ml $^{45}CaCl_2$ solution which was put in a circular lucite tray with the surface area of 12.56 cm^2. The β radiation from the aqueous surface was measured by an end window Geiger–Müller tube (Amperex, 200 HB, about 1.4–2.0 mg/cm^2) held close to the surface at a distance of 2 mm. In order to avoid disturbances, the collimator was used to eliminate the radiation from the brim of the aqueous surface. An electronic counter (Nuclear Chicago, Model 8735, Chicago, Ill.) with a chronographic digital printer was used together with a high-voltage supply (Technical Measurement Corporation, North Haven, Conn.) to record the change in surface radioactivity. The bulk count rate, which is the surface count without a monolayer, was subtracted from the total count rate observed at the monolayer surface to give the amount of Ca^{2+} adsorbed. The factor used for converting the radioactive count rate to the amount of Ca^{2+} adsorbed was obtained in the following way. Various known amounts of radioactive $CaCl_2$ solution were placed on circular lucite plates, which have the same area as the aqueous surface, in more than 300 drops as homogeneous as possible using a microsyringe. The radioactivities from the plates were counted under the same geometrical condition as in the adsorption measurement after drying them in a desiccator. The back scattering coefficient of ^{45}Ca β-rays by the lucite plate was shown to be almost the same as that of water by preliminary experiment.

RESULTS AND DISCUSSION

The calibration curve thus obtained between radioactive count rate (cpm) and amount of calcium on the surface Γ_{Ca} (moles/cm^2) showed a good straight line as shown in Fig. 1. The slope of the curve gives the conversion factor. The conversion factors used in this experiment were justified by measuring the adsorption of Ca^{2+} on a stearic acid monolayer, for which the binding characteristics of Ca^{2+} are



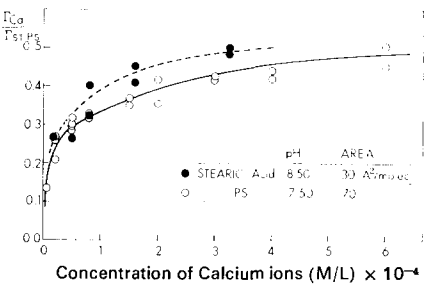


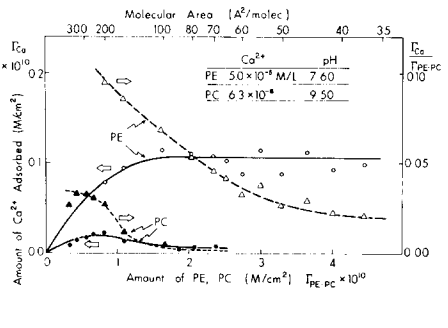
Fig. 1. Calibration curve for radioactive calcium.

Fig. 2. Adsorption of Ca^{2+} on phosphatidylserine (PS) and stearic acid monolayers.

relatively well known, as shown in Figs 2 and 6. The maximum binding ratio of Ca^{2+} to stearic acid Γ_{Ca}/Γ_{ST} in this measurement turned out to be 0.5, which agreed well with those values found by other investigators[37–43], indicating two point electrostatic attachment of Ca^{2+} to the stearate ions. The pH shown in Fig. 2 was so chosen that all the stearic acid in monolayer was expected to have an ionic form and could react with Ca^{2+} to form a metallic soap, as shown in the literature[39,42]. This agreement might indicate that our whole experimental procedures were correct and reliable. The equilibrium of adsorption was reached in less than an hour in almost all cases during the present adsorption measurements. At room temperature, within such a short length of time, the loss of the monolayers into the supporting solution by desorption is expected to be negligibly small. This has been confirmed by the previous study of desorption[44] of phosphatidylserine monolayer which might be the most soluble lipid employed in the present experiment.

Accessibilities of Ca^{2+} to anionic sites in phospholipid monolayers

In a previous paper[45] we have reported on the different accessibilities of Ca^{2+} to the different anionic site of phospholipids, in which the amounts of calcium bound to the lipid monolayer Γ_{Ca} were shown in relation to the molecular packing of the lipids Γ_{lip} together with the binding ratio of Ca^{2+} to the lipid molecules, $R=\Gamma_{Ca}/\Gamma_{lip}$. For stearic acid monolayer, Γ_{Ca} increases proportionally with Γ_{ST}, and the linear relation passes through the origin. Thus, R is constant for stearic acid, irrespective of the increase of Γ_{ST}, which means that the Ca^{2+} is always accessible to the carboxyl group at all degrees of molecular packing of stearic acid. For the phosphatidylethanolamine and phosphatidylcholine monolayers in Fig. 3, the Γ_{Ca} curves showed saturation with molecular packing of phosphatidylethanolamine or phosphatidylcholine higher than 150 Å^2/molecule, which means the decrease of binding ratio R with the increase of Γ_{PE} or Γ_{PC}. There is no site for Ca^{2+} to bind with in phosphatidylethanolamine or phosphatidylcholine molecules, other than with the phosphate group. It might be reasonable at present to explain the saturation phenomena as being an indication of changed accessibility of Ca^{2+} to the phosphate group as a result of a conformational change of the lipid molecules in the monolayer. The monolayer of phosphatidylserine, which has two anionic groups of phosphate and carboxyl, showed an isotherm resembling the additive form of both stearic acid



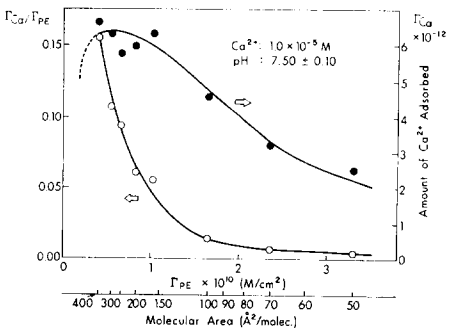


Fig. 3. Effect of molecular packing in phosphatidylethanolamine (PE) and phosphatidylcholine monolayers on Ca^{2+} adsorption.

Fig. 4. Effect of molecular packing in phosphatidylethanolamine (PE) monolayers on Ca^{2+} adsorption.

and phosphatidylethanolamine type isotherms. The Γ_{Ca} increased almost linearly with Γ_{PS}, but showed a tendency for a slight upward shift (refer to similar curve in Fig. 5). The linear part of the curve did not pass through the origin, thus differing from that of stearic acid. This indicates that the adsorption of Ca^{2+} occurred mainly on the carboxyl group and partly on the phosphate group of the phosphatidylserine monolayer. In Fig. 4, further confirmation of the limited access of Ca^{2+} to the phosphate group of phosphatidylethanolamine is shown. Here, the radioactive $^{45}Ca^{2+}$ was injected into the substrate solution beneath the phosphatidylethanolamine monolayer instead of spreading a monolayer on the radioactive solution as in the previous experiment. The adsorption of Ca^{2+} was measured after the equilibrium condition was reached by slowly stirring the substrate solution using a magnetic stirrer. The change of accessibility can be seen more clearly in this figure. The Γ_{Ca} decreases with the increase of Γ_{PE}, in spite of the increase in the total number of anionic site per unit area. The binding ratio R shows the change of accessibility more markedly. It decreases sharply with Γ_{PE} and approaches almost zero around 80 $Å^2$/molecule packing of the lipid.

Similar effects of molecular conformation on the chemical reactivities of the lipid monolayer are well known[46,47] for the oxidation of the monolayer of unsaturated fatty acid spread on aqueous $KMnO_4$ solution[48–55], for the hydrolysis of lecithin or ester monolayer by snake venom[55], or by some enzymes[57,58], and for the lactonization[59] of γ-hydroxystearic acid monolayer. Hauser and Dawson[59] also found for phosphatidylinositol monolayers that the displacement of bound Ca^{2+} by alkali metal ions or by some local anesthetics was affected by the molecular packing of the lipid[60,61].

The combined feature of the two types of isotherm for phosphatidylserine monolayers, mentioned above, is also seen in Fig. 5 in which the monolayer consists of both phosphatidylethanolamine and phosphatidylserine in a 3:1 ratio, and shows the upward shift more clearly than the experiment in the previous report[45]. The dilution of the phosphalidylserine monolayer with phosphatidylethanolamine decreases the number of carboxyl groups in the monolayer compared with that of the phosphate to which the accessibility of the Ca^{2+} is limited. The greater shift of the isotherm in this figure might also support the additive relation.

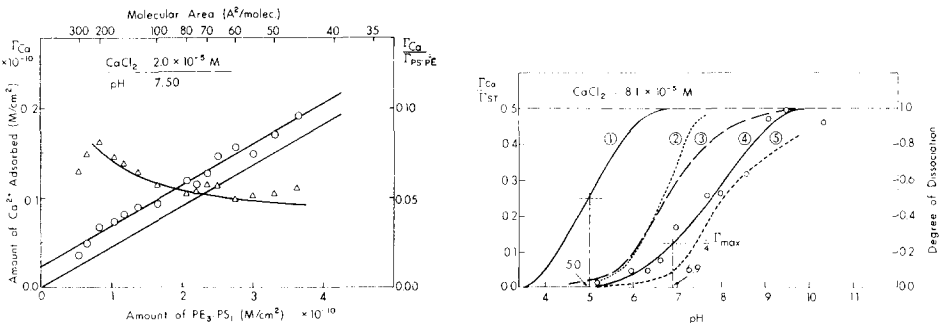


Fig. 5. Adsorption of Ca^{2+} on 3:1 mixed monolayers of phosphatidylethanolamine (PE) and phosphatidylserine (PS).

Fig. 6. Effect of pH on calcium adsorption to stearic acid monolayers. 1, bulk dissociation curve; 2, Sobotka[38] and Ellis[39]; 3, Matsubara[41]; 4, ours; 5, Bagg[42].

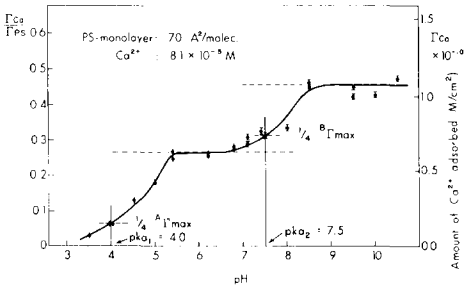
The amount of calcium bound to the lipids varies in the order phosphatidylserine > stearic acid > phosphatidylethanolamine ≫ phosphatidylcholine at pH 7.5 and with a calcium concentration of $5 \cdot 10^{-5}$ M.

Effect of pH on calcium binding

The amount of adsorption of Ca^{2+} was measured at various pH values for stearic acid, phosphatidylserine and phosphatidylethanolamine as shown in Figs 6, 7 and 8.

The curve obtained for stearic acid (Fig. 6) closely resembled those curves obtained by many other investigators in different ways[37–41]. The agreements of the shape of our isotherm, and the absolute amount of bound calcium or the binding ratio (Figs 2 and 5) with those found in literatures also support the present experimental procedures and calibration. The Ca^{2+} and stearic acid monolayers are considered to form di-soap[41,42] at the saturation point of adsorption by the so-called two-points electrostatic attachment[61]. The formation of the basic soap might not be the case at such extremely low concentrations of Ca^{2+} and at pH values lower than 12[62]. Infrared absorption spectroscopy by Ellis and Pauley[39] also confirmed that there was no incorporation of the basic salt in the stearic acid monolayers spread on an aqueous $CaCl_2$ solution at pH values below 8.

Figs 7 and 8 show the similar pH effect on the Ca^{2+} binding for phosphatidylserine and phosphatidylethanolamine monolayers. In order to make the subsequent analysis easier, we chose the molecular areas of 70 Å²/molecule for phosphatidylserine and 150 Å²/molecule for phosphatidylethanolamine. They are presumed to be the areas at which Ca^{2+} is not accessible to the phosphate group of phosphatidylserine, but can react with carboxyl group of phosphatidylserine and with the phosphate group of phosphatidylethanolamine. The curves show a very characteristic stepwise feature, and agree well with the results obtained by Rojas and Tobias[13], although their binding ratios show very high value of 2 for phosphatidylserine, and 1 for phosphatidylethanolamine. Our curves also resemble those curves obtained by Joos and Carr[20] from their chelate titration data of Ca^{2+} in equilibrium with the phospholipid suspension separated by a dialysis membrane. Their binding ratio



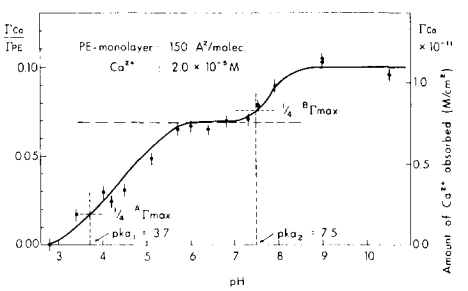


Fig. 7. Effect of pH on Ca^{2+} adsorption to phosphatidylserine (PS) monolayers.

Fig. 8. Effect of pH on Ca^{2+} adsorption to phosphatidylethanolamine (PE) monolayers.

agreed well with ours, and gave the maximum values of 1.0 for phosphatidylserine and 0.5 for phosphatidylethanolamine, which can reasonably be expected from the simple stoichiometry of electrostatic interaction.

It seems most likely that the stepwise features are due to the well known ampholytic nature of these phospholipids, and of the inter- or intramolecular salt formation, as discussed in the following section. Similar stepwise changes in the physico-chemical properties are also observed for phosphatidylethanolamine, phosphatidylcholine and phosphalidylserine surface potential data, by Papahadjopoulos[31], and also by Standish and Pethica[24]. They are shown together in Fig. 8.

The estimation of surface pK_a values of ionic groups, and intermolecular salt formation

The ionic equilibrium for the dissociation of acid as shown in Reaction 1 may be expressed by the well-known mass action relation, Eqn 2.

$$HA \overset{k}{\rightleftharpoons} H^+ + A^- \tag{1}$$

$$pK = pH + \log \frac{[HA]}{[A^-]} = pH + \log \frac{[1-\alpha]}{[\alpha]} \tag{2}$$

The constant α expresses the degree of dissociation, and the brackets stand for the activities of each species which are conveniently expressed as concentration units in the present calculation, assuming the activity coefficient to be equal to unity. The acid dissociation constant, pK_a, is usually estimated as the pK of half ionization of the acid or base at which $[HA]=[A^-]$ or $[1-\alpha]=[\alpha]$. If we express the total amount of acid as $[R]$, $[R]=[A^-]+[HA]$, then pH is equal to pK at $[A^-]=\frac{1}{2}[R]$.

If we can assume that the calcium binding is the di-soap formation, and assume the same equilibrium constant $k'=k''$ for Reactions 3 and 4, shown below,

$$Ca^{2+} + A^- \overset{k'}{\rightleftharpoons} CaA^+ \tag{3}$$

$$CaA^+ + A^- \overset{k''}{\rightleftharpoons} CaA_2 \tag{4}$$

the concentration of the bound calcium $[CaA_2]$ should be proportional to the square concentration of the dissociated acid when the concentration of Ca^{2+} is constant.

$$[CaA_2] = k[Ca^{2+}][A^-]^2 = \gamma[A^-]^2 \tag{5}$$

Here, γ represents the overall binding constant. In this system we can expect the maximum calcium binding to be

$$[CaA_2]_{max} = \gamma[R]^2 \tag{6}$$

At the condition when $pK = pH$, the amount of bound calcium $[CaA_2]$ will be

$$[CaA_2] = \gamma[\tfrac{1}{2}R]^2 = \tfrac{1}{4}\gamma[R]^2 = \tfrac{1}{4}[CaA_2]_{max} \tag{7}$$

This means that the pH which gives 1/4 of the maximum adsorption Γ_{Ca}^{max} expected at the given concentration of calcium, and also with a given amount of acid in the monolayer, might represent the pK value of the corresponding acid. The surface pK obtained for stearic acid in Fig. 6 is 6.9, two pH units higher than the value obtained as a mid point of the bulk dissociation curve shown in the same figure. The shift of the pK_a value of the acid at the surface to a value higher than that in the bulk is a common tendency for charged monolayers[38]. Such shifts have been observed by many people in various measurements[64,72], which we will further mention later together with the pK values of phospholipid.

In order to explain the following two experimental observations, it is convenient to assume that there are ionic equilibria among those ions of ampholyte, hydrogen and Ca^{2+}, as diagramatically shown in Fig. 10. They are, (i) the decreased net reactivity of the phospholipid to Ca^{2+} in the order phosphatidylserine > phosphatidylethanolamine ≫ phosphatidylcholine, even though these all have the same phosphate group to react with Ca^{2+} and (ii) the stepwise features of the adsorption isotherm for phosphatidylserine and phosphatidylethanolamine as shown in Figs 7, 8 and 9. The reactivity may be compared through the amounts of calcium bound to the lipid monolayers at the same molecular area, and at the same concentration of Ca^{2+} and H^+. In Fig. 10, the phospholipids, phosphatidylethanolamine and phosphatidylserine, are represented only by their ionic part. The small circles to the left of the ionic symbols express the other parts of the phospholipid, and the equilibrium constants k_1–k_7 for those equilibria are indicated by arrows. The equilibria

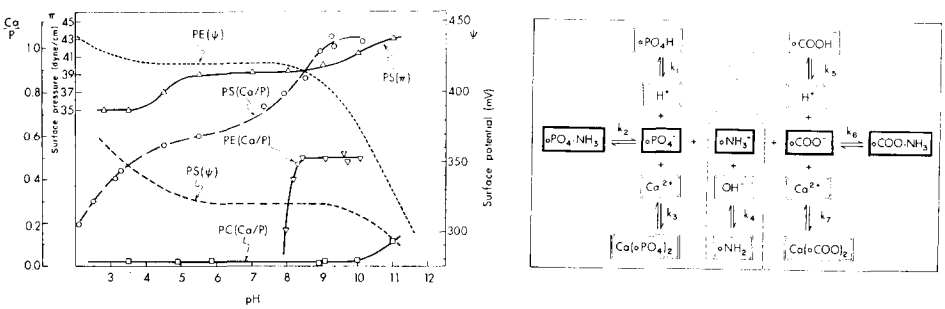


Fig. 9. Effect of pH on the physicochemical properties of phospholipid. Surface potential (↓) and pressure (π); by Papahadjopoulos[31], Ca^{2+} binding ratio (Ca/P); by Joos and Carr[20].

Fig. 10. Equilibria among the ionic groups of phospholipid, Ca^{2+} and H^+.

involve the neutralization of the electrostatic charges of both the anionic and the cationic groups of the molecule. This might occur in either inter- or intramolecular ways, which means that the ammonium group of phospholipid can form a molecular salt with either the phosphate or carboxyl group in the same molecule or in an adjacent molecule. The neutralization between the carboxyl and the amino groups of phosphatidylserine might not be an intramolecular type, because these two groups are directed against each other for L-serine. This may also be called a competition between ammonium and Ca^{2+} for binding with the anionic sites of phospholipid[53].

Intermolecular salt formation for phosphatides has been suggested by many investigators, Kuhn *et al.*[63] from their dielectric constant measurement, Bear *et al.*[64], Bevan and Malkin[65], Finean[66] and Dervichian[67] from their X-ray crystallographic analyses of packing, and Pethica[68], Shah and Schulman[69] from their surface potential measurements. The ampholytic surface-active agents like dodecyl betain, or β-alanine type compound are also well known for their non-ionic behavior[72,73] and low reactivities to heavy metal ions in water[74]. The negative charge of the phosphate or the carboxyl group may be shielded from the access of Ca^{2+} by the positive amino group or by the formation of a neutral ammonium phosphate or ammonium carboxylate salt to a variable extent according to the ionic nature of ammonium group and also to the molecular structure and conformation of the lipid.

At pH values lower than 3, in Fig. 8 or 9, nearly all the anionic group will be present as a free acid form, and there may be few anions available for Ca^{2+} to bind with. But, as the pH rises, the $-PO_4H$ or $-COOH$ groups will dissociate into free $-PO_4^-$ or $-COO^-$ anions as determined by their pK_a values. Some of these can react with $-NH_3^+$ to form $-PO_4 \cdot NH_3$ or $-COO \cdot NH_3$ salts, and others can react with Ca^{2+}. This stage of the isotherm parallels the dissociation curve of PO_4H and/or COOH. Thus, the pH that gives the 1/4 of maximum adsorption increment at the acid side (pH from 3 to 6), $1/4\ \Gamma_{Ca}^{max}$, should represent the pK value of the acid group concerned.

In the pH region from 5.5 to 7.0, where ampholytic phospholipids are generally recognized to take the zwitterionic form, no change will occur in calcium binding as the pH is changed. This can be observed in Figs 7, 8 and 9, where all the acids and bases are fully dissociated to form free ions and internal salts.

By increasing the pH above 7, so as to affect the dissociation of amino group by the equilibrium K_4, a certain amount of $-PO_4^-$ and/or $-COO^-$ anion, with which Ca^{2+} can react, will be released from the salts $-PO_4 \cdot NH_3$ or $-COO \cdot NH_3$ owing to the decrease of the equilibrium $-NH_3^+$ concentration. Thus, the adsorption isotherm of Ca^{2+} at this pH region reflects directly the dissociation of the $-NH_2$ group, and the pH that gives the adsorption of 1/4 of maximum increment at the alkaline side (pH from 6 to 9), $1/4\ \Gamma_{Ca}^{max}$, should represent the pK value of the base group concerned.

In the pH region high enough to change all the $-NH_3^+$ group into an undissociated form $-NH_2$, the degree of calcium binding is determined only by the Ca^{2+} concentration, the binding constants of calcium k_3 and k_7, and the total amount of phospholipid. As long as the total amount of lipid and the activity of the Ca^{2+} are kept constant, and assuming the equilibrium constants to be constant at all pH values, no change is expected for calcium adsorption at pH values higher than the pK value of the corresponding ammonium group. By applying the law of mass action

to the ionic processes in Fig. 10, we can express each equilibrium constant, from k_1 to k_7 as in Eqns 8–14.

$$k_1 = \frac{[\text{–PO}_4\text{H}]}{[\text{–PO}_4^-][\text{H}^+]} \tag{8}$$

$$k_2 = \frac{[\text{–PO}_4\cdot\text{NH}_3]}{[\text{–PO}_4^-][\text{–NH}_3^+]} \tag{9}$$

$$k_3 = \frac{[\text{Ca(–PO}_4)_2]}{[\text{Ca}^{2+}][\text{–PO}_4^-]^2} \tag{10}$$

$$k_4 = \frac{[\text{–NH}_3^+]}{[\text{–NH}_2][\text{H}^+]} \tag{11}$$

$$k_5 = \frac{[\text{–COOH}]}{[\text{–COO}^-][\text{H}^+]} \tag{12}$$

$$k_6 = \frac{[\text{–COO}\cdot\text{NH}_3]}{[\text{–COO}^-][\text{–NH}_3^+]} \tag{13}$$

$$k_7 = \frac{[\text{Ca(–COO)}_2]}{[\text{Ca}^{2+}][\text{–COO}^-]^2} \tag{14}$$

The total concentration of phospholipid $[\Gamma_{\text{lip}}]$ involved in these reactions is expressed by Eqns 15 and 16.

$$[\Gamma_{\text{lip}}] = [\text{–PO}_4\cdot\text{NH}_3] + [\text{–PO}_4^-] + [\text{–PO}_4\text{H}] + 2[\text{Ca(–PO}_4)_2] \tag{15}$$

$$= [\text{–PO}_4\cdot\text{NH}_3] + [\text{–NH}_3^+] + [\text{–NH}_2] \tag{16}$$

The amount of total lipid Γ_{lip}, and the amount of bound calcium Γ_{Ca} can be obtained experimentally as the 2-dimensional quantities, expressed as moles/cm^2 of surface layer, but are conventionally converted here to a 3-dimensional concentration, as is observed above, by assuming the thickness of the surface layer to be equal to the characteristic distance of ionic atmosphere defined by the Debye-Hückel equation (Eqn 17)[75].

$$\frac{1}{\kappa} = \left(\frac{1000\,DKT}{4\pi\varepsilon^2 N \Sigma C_i Z_i^2}\right)^{\frac{1}{2}} \tag{17}$$

Here, κ indicates the reciprocal length of the ionic atmosphere of Debye and Hückel, ε the electronic charge, N the Avogadro's number, C_i and Z_i the concentration and the valency of each ionic species. D the dielectric constant of water, K the gas constant

and T the absolute temperature. We used in the present calculation the κ values obtained for $CaCl_2$ solution at experimental concentrations.

The equilibrium constants K_2, K_3, K_6 and K_7 were tentatively calculated by solving Eqns 8–16 simultaneously for the experimental values of adsorption at both pH 7 and 9, and the pK values obtained graphically from Figs 8, 9 and 10, and all are summarized in Table II. The surface pK_a values are seen to be about 2 pH units larger than the bulk pK_a for the acid as previously stated for stearic acid, and about

TABLE II

IONIC CONSTANTS OF PHOSPHOLIPIDS

Lipid	*Ionic group*	pK_a $-log[H^+][A^-]/[HA]$ or $-log[H^+][B]/[B^+]$		*Binding constant of* Ca^{2+} $[CaA_2]/[Ca^{2+}]\cdot[A^-]^2$	*Equilibrium constant of molecular salt formation* $[AB]/[A^-][B^+]$	
		Bulk	*Surface*			
Stearic acid	–COOH	5.00	6.9	$3.6\cdot10^5$		
Phosphatidyl-L-serine (70 Å²/-molecule)	–COOH	2.21	4.0	$2.92\cdot10^6$	$COO\cdot NH_3$	$7.62\cdot10$
	$-NH_2$	9.15	7.5	—		
	$-PO_4H$	1.40	3.7*	$6.41\cdot10^5$*	$PO_4\cdot NH_3$	$5.61\cdot10$*
Phosphatidyl-ethanolamine (150 Å²/-molecule)	$-NH_2$	9.15	7.5	—	$PO_4\cdot NH_3$	$5.61\cdot10$
	$-PO_4H$	1.40	3.7	$6.41\cdot10^5$		
Phosphatidyl-choline	$-N(CH_3)_3$		(11.6)**	—	$PO_4\cdot N(CH_3)_3$	$8.85\cdot10^2$
	$-PO_4H$	1.40	3.7*	$6.41\cdot10^5$*		

* Not measured, but regarded to be the same as that of phosphatidylethanolamine in the present analysis.

** Calculated from the experimental data by Joos and Carr[20].

1.7 units smaller for the base. The change of the pK value at the surface from that of the bulk is well known for fatty acids and amine monolayers (Peters[74]). It is also known as a cause of the surface hydrolysis phenomena, and is explained on the basis of Gouy's[75] theory as an effect of surface charge on the concentration of the counter ions in the surface layer[7,79]. The bulk pK values of –COOH, $-NH_2$ and $-PO_4H$, in the third column of Table II, are taken from the values for L-serine[80], and the first pK_a value of α-glycerophosphoric acid[81]. Due to the technical restrictions of accuracy in radiometry, we did not have the pH–Γ_{Ca} relation for phosphatidylcholine, and therefore, the surface pK_a value for the $-N(CH_3)_3$ group of phosphatidylcholine was calculated from the adsorption isotherm obtained by Joos and Carr[20]. The pK_a values obtained by other investigators using the different methods of measurement for these phospholipids are shown in the 4th and 5th column of

Table III. The values listed in the 4th column are calculated by applying our treatment to the adsorption data obtained by Rojas and Tobias[13] and Joos and Carr[20].

The binding constants found in the literature[82–84] are listed in Table IV, together with our values. The literature values express the apparent binding constant which assumes 1:1 binding between Ca^{2+} and lipid. No distinction was made for the distribution of calcium between $-PO_4^-$ and $-COO^-$. The large disagreement

TABLE III

SURFACE pK VALUES

Lipid	*Ionic group*	*Surface* pK_a		*Literature*
		The calculation applied to:		
		Our date	*Data in literature*	
Stearic acid		6.9	6.2–7.4 (refs 37–40)	
Phosphatidylserine	–COOH	4.0	3.7 (ref. 11) 2.0 (ref. 19)	4.42*, 5.5**
	$-NH_2$	7.5	8.0 (ref. 12)	9.93*, 9.7**
	$-PO_4H$	3.7	—	
Phosphatidylethanolamine	$-NH_2$	7.5	8.0 (ref. 12)	10.45***
	$-PO_4H$	3.7	4.5 (ref. 12)	0.32***
Phosphatidylcholine	$-N(CH_3)_3$	—	11.6 (ref. 19)	11.6**
	$-PO_4H$	3.7	—	5.5**

* Apparent pK (no distinction between $-PO_4$ and –COOH) obtained by titration[81].
** Apparent pK calculated from the conductance data of the bilayer[80].
*** Apparent pK calculated from surface potential data[24].

TABLE IV

BINDING CONSTANT OF Ca^{2+}

Lipid	*Ionic group*	*Binding constant of* Ca^{2+}	
		Ours	*Literature*
Stearic acid	$-COO^-$	$3.6 \cdot 10^5$	
Phosphalidylserine	$-COO^-$	$2.9 \cdot 10^6$	$1.17 \cdot 10^4$*, $1.4 \cdot 10^7$**, 10^7***
	$-PO_4^-$	$6.4 \cdot 10^5$	
Phosphatidylethanolamine	$-PO_4^-$	$6.4 \cdot 10^5$	$6 \cdot 10^6$***
Phosphatidylcholine	$-PO_4^-$	$6.4 \cdot 10^5$	10^6***

* Apparent binding constant ($Ca \cdot lip/Ca^{2+}$ lip^{2-} calculated from electrophoresis data of lipid suspension[18].
** Apparent binding constant obtained by radiotracer measurement for monolayer[60].
*** Apparent binding constant obtained by the potentiometric titration of lipid suspension[82].

of their values from ours comes not only from the assumption of 1:1 binding but also from the different estimation of the volume of water involved in this ionic process.

Considerable ambiguity is still left in the present analysis, that is, (i) the equilibrium constants for these ionic processes should depend largely on the concentration of each ionic species, and those values obtained in the present analysis only stand for the apparent constant, and are meaningful at the particular conditions of the present experiments, (ii) the concentrations which appeared in the mass equation all have to be the activities instead of the conventional analytical concentrations, as used in the present calculation, (iii) the amount of water involved in these ionic processes is an unknown factor, and use of the reciprocal length of the ionic atmosphere as a thickness of the surface layer might give a close estimate of the actual system but not the strict one.

In spite of these great difficulties, the authors still feel that the present tentative analysis is significant as a small step in revealing the complex ionic structure and functions of the biological membrane.

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