The ionic structure of lecithin monolayers

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ABSTRACT Surface potentials of mixed monolayers of dicetyl phosphate and eicosanyl trimethylammonium bromide (1:1) were the same on subsolutions of 0.02 M NaCl or 0.01 M $CaCl_2$, which indicated that ionic phosphate does not interact with Ca^{++} in the presence of a neighboring trimethylammonium group. Surface potential–pH plots of dicetyl phosphate, and of dipalmitoyl, egg, and dioleoyl lecithins showed that as the pH of the subsolution is decreased the phosphate groups in the monolayer are neutralized in the order: dicetyl phosphate > dipalmitoyl lecithin > egg lecithin > dioleoyl lecithin.

The binding of cations (Na^+, Ca^{++}) to the phosphate group of lecithin also showed the same order. The binding of Ca^{++} to egg phosphatidic acid monolayers, as measured by the increase in surface potential, is considerably greater than that to egg lecithin.

These results suggest that there is an *internal salt linkage* between the phosphate and trimethylammonium groups on the same lecithin molecule. An increase in unsaturation of fatty acyl chains increases the intermolecular spacing, which reduces the ionic repulsion between polar groups, and hence strengthens the internal salt linkage. The results support the concept of a vertical rather than coplanar orientation of the phosphoryl choline group with respect to the interface. A position has been proposed for Ca^{++} in the dipole lattice of lecithin from a consideration of the surface potential measurements.

KEY WORDS dicetyl phosphate · lecithin (dioleoyl, egg, dipalmitoyl) · surface potential–pH · surface potential–log (salt concn) · internal salt linkage · strength of the salt linkage · calcium binding · effect of cholesterol and unsaturation · position of calcium

LECITHIN IS GENERALLY the major fraction of the total phospholipid occurring in biological membranes. Since the lecithin molecule possesses a phosphate and a trimethylammonium group separated by two methylene groups, its structure allows two ionic forms: one in which the separation of charges is maximal and the other in which a reduced separation of charges results from an internal salt linkage between the phosphate and trimethylammonium groups in the same molecule. These ionic charges are of considerable interest in relation to lipid–protein interaction and to ionic transport in the membrane. This paper describes a surface chemical investigation of the ionic charges in lecithin monolayers, and the influence of calcium, cholesterol, and the unsaturation of fatty acyl chains upon these charges.

MATERIALS

The dipalmitoyl lecithin, dicetyl phosphate, egg lecithin, egg phosphatidic acid and cholesterol were the same as those described in the accompanying paper (1). Eicosanyl trimethylammonium bromide was purchased from K & K Laboratories Inc. (Plainview, N.Y.). Dioleoyl lecithin, which was further purified by thin-layer chromatography, was a gift from Dr. L. L. M. Van Deenen.

METHODS

Surface Pressure and Surface Potential Measurements

The methods of measuring surface pressures by means of a modified Wilhelmy plate, and surface potentials by a radioactive electrode have been described previously (2). Surface pressures and surface potentials of lecithin monolayers were measured on subsolutions of NaCl as well as $CaCl_2$ (10^{-1}M–10^{-4}M) at pH 5.6 and 25°C. Surface measurements were also made on subsolutions of citric acid–sodium citrate buffer (μ = 0.05) at pH 2–6, and Tris–HCl buffer (μ = 0.05) at pH 7–9. Inorganic chemicals of reagent grade and twice-distilled water were used.

RESULTS AND DISCUSSION

Dicetyl Phosphate–Eicosanyl Trimethylammonium (1:1) Monolayers

Fig. 1 shows the surface pressure–area and surface potential–area curves of mixed monolayers of dicetyl phos-

phate and eicosanyl trimethylammonium in molar ratio 1:1 on subsolutions of 0.02 M NaCl and 0.01 M $CaCl_2$, at pH 5.6 and 25°C. It is known (3) that alkyl trimethylammonium forms expanded monolayers because of the ionic repulsion between molecules. However, the presence of anionic phosphate groups in the mixed monolayers reduces the ionic repulsion, and thereby results in a condensed film, as indicated by the steep surface pressure–area curve (Fig. 1).

The surface potentials of the mixed monolayers are the same on subsolutions of NaCl or $CaCl_2$, which indicates that the ionic phosphate group does not interact with calcium in the presence of neighboring trimethylammonium groups. Because of the formation of salt linkages in the monolayer lattice, such mixed monolayers exhibit properties similar to those of nonionic monolayers (4, 5). In contrast to the gaseous or liquid state of alkyl trimethylammonium monolayers, as inferred from the mobility of sprinkled talc particles, the mixed monolayers are in the solid state above a surface pressure of 4–5 dynes/cm. It is stressed here that the solidification of the mixed monolayer is caused primarily by salt linkages and to a lesser extent by saturated hydrocarbon chains, since a solid monolayer does not retain its solidity when molecules that form liquid monolayers are added to it, despite the interaction between hydrocarbon chains.

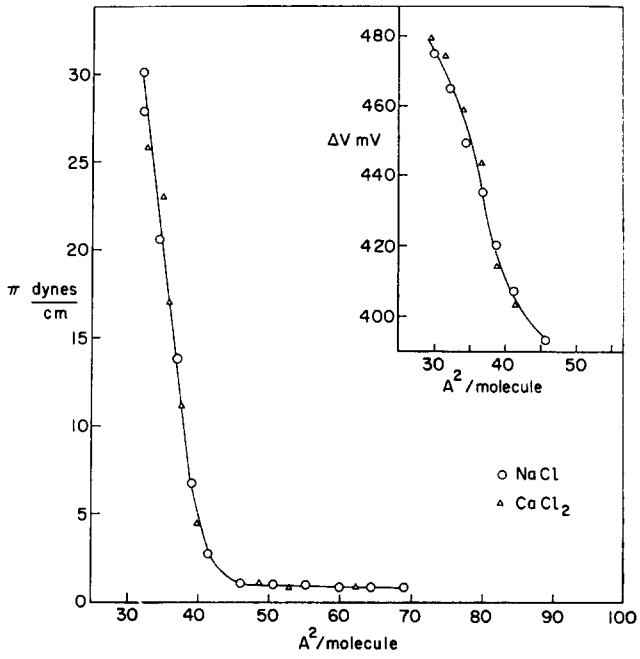

FIG. 1. Surface pressure–area and surface potential–area curves of mixed monolayers of dicetyl phosphate and eicosanyl trimethylammonium (1:1) on subsolutions of 0.02 M NaCl (○) or 0.01 M $CaCl_2$ (△), at pH 5.6 and 25°C.

Ionic Structure of Lecithin

We have reported (2) that the degree of interaction of lecithin monolayers with Ca^{++} decreases with the degree of unsaturation of fatty acyl chains. This result cannot be explained on the basis of intermolecular spacing alone since dioleoyl phosphate and dioleoyl lecithin monolayers, which have a similar intermolecular spacing, showed strikingly different interaction with calcium ions.[1] Thus we are led to the conclusion that the trimethylammonium and phosphate groups of a dioleoyl lecithin molecule form an internal salt linkage, which prevents the interaction of the phosphate group with Ca^{++}.

Fig. 2 A represents monolayers of dioleoyl, egg, and dipalmitoyl lecithins and their interaction with Ca^{++} by means of Fisher-Hirschfelder-Taylor scale models. On the basis of the interaction of metal ions with lecithin monolayers (2) and results to be presented later in this paper, we propose the ionic structure of lecithin schematically illustrated in Fig. 2 B. The progressive weakening of the internal salt linkage with increasing saturation of the fatty acyl chains (due to increase in ionic repulsion between similar charges), and the concomitant increased binding of calcium, is represented in the diagrams (Fig. 2 B). It should be stressed that Fig. 2 A–C is a static representation of average kinetic states of molecules at the interface and is meant to illustrate the increase in *effective* molecular area with increase in unsaturation of fatty acyl chains.

Influence of Cholesterol on Ionic Structure of Egg Lecithin

We have previously shown (1) that the presence of 25 moles %, or more, of cholesterol in mixed monolayers prevents the binding of Ca^{++} to egg lecithin. A comparison of Fig. 2 C with the middle diagram of Fig. 2 B illustrates the explanation of the effect of cholesterol on the interaction of egg lecithin with calcium. In egg lecithin, the phosphate group interacts with a calcium ion and also with the adjacent trimethylammonium group (Fig. 2 B). The presence of cholesterol causes (statistically) a small net increase in the spacing between phosphate groups which reduces the ionic repulsion between the polar groups and strengthens the internal salt linkage (Fig. 2 C). Consequently, mixed monolayers of egg lecithin–cholesterol do not bind Ca^{++}. Although addition of cholesterol to egg lecithin monolayers does not cause *proportional* increase in area/molecule, it does change the spacing between adjacent phosphate groups because of a decrease in the surface concentration of egg lecithin.

[1] Work done recently in this laboratory by Mr. Bhupendra Shah (unpublished) showed that the surface potential of dioleoyl phosphate and dioleoyl lecithin monolayers increases by 100 mv and 5 mv, respectively, when 0.01 M $CaCl_2$ is substituted for 0.02 M NaCl in the subsolution.

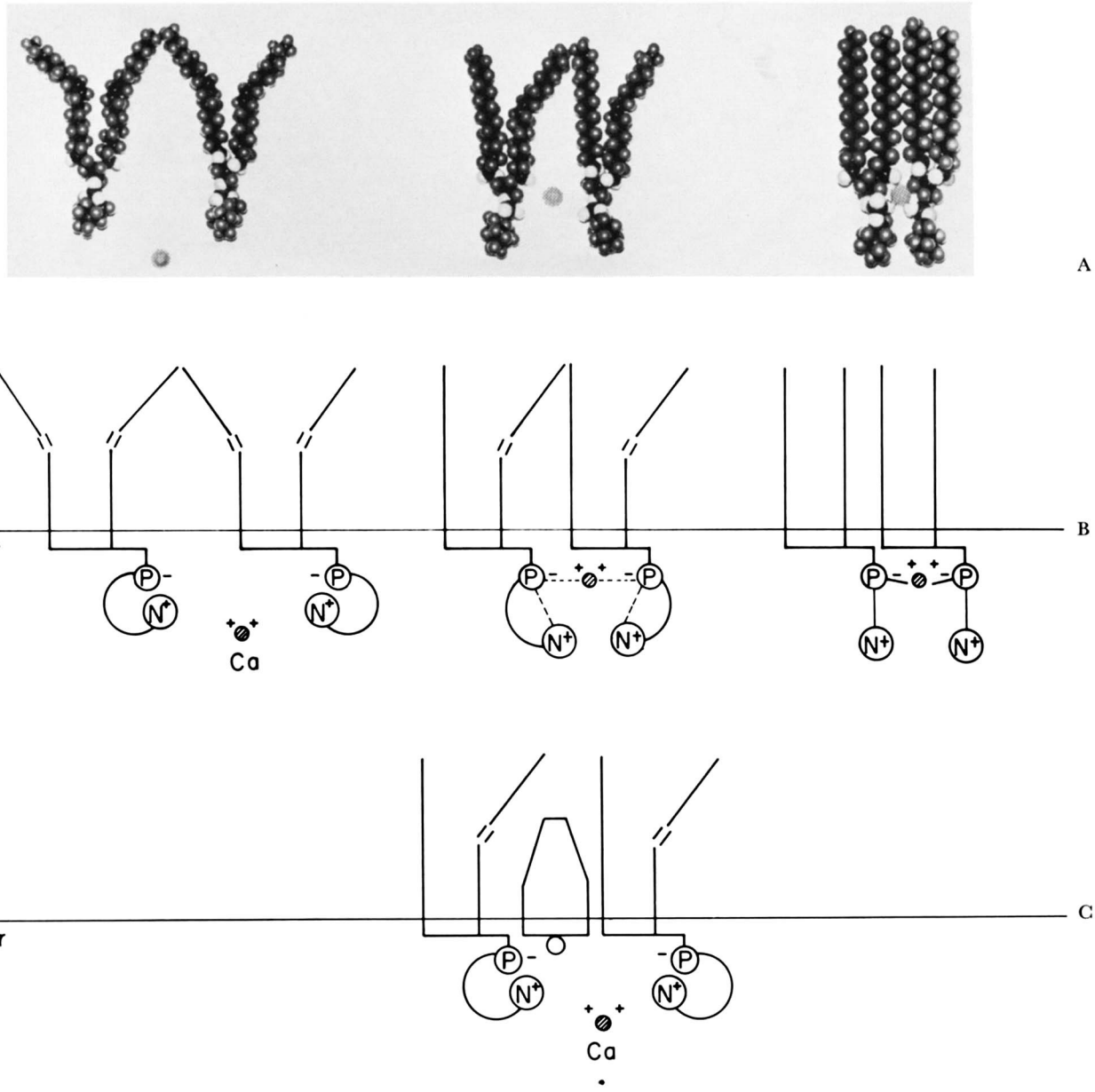

FIG. 2. (*A*) Fisher-Hirschfelder-Taylor scale models of dioleoyl, egg, and dipalmitoyl lecithins. The stippled sphere represents calcium ion. (*B*) A schematic representation of interaction of calcium ion with corresponding lecithins. Dioleoyl lecithin shows an internal salt linkage between the phosphate and trimethylammonium groups. The broken lines in the egg lecithin diagram represent weak interactions of the phosphate with Ca^{++} and with the trimethylammonium group. The solid line between Ca^{++} and the phosphate group in the dipalmitoyl lecithin diagram represents a strong interaction. (*C*) A schematic representation of egg lecithin–cholesterol monolayers in which the increased spacing between phosphate groups results in a strong internal salt linkage, which prevents the binding of Ca^{++}.

Influence of pH on Surface Pressure–Area Curves

The surface pressure–area curves of dicetyl phosphate and of dipalmitoyl, egg, and dioleoyl lecithin have been reported previously (2, 6). They were not altered over the pH range 2–8. This agrees with the results reported by Anderson and Pethica (7) that the surface pressure–area curves of distearoyl lecithin monolayers were the same throughout the pH range 1–7.

Surface Potential–pH Curves

For investigation of the ionic properties of a monolayer, its surface potential at a fixed value of area/molecule is plotted against pH of the subsolution; this is generally called the ΔV–pH curve of the monolayers. It has been shown by Schulman and Hughes (8) that the ΔV–pH curve of a fatty acid resembles its titration curve except for a shifted pK value due to the "surface pH" effect. For

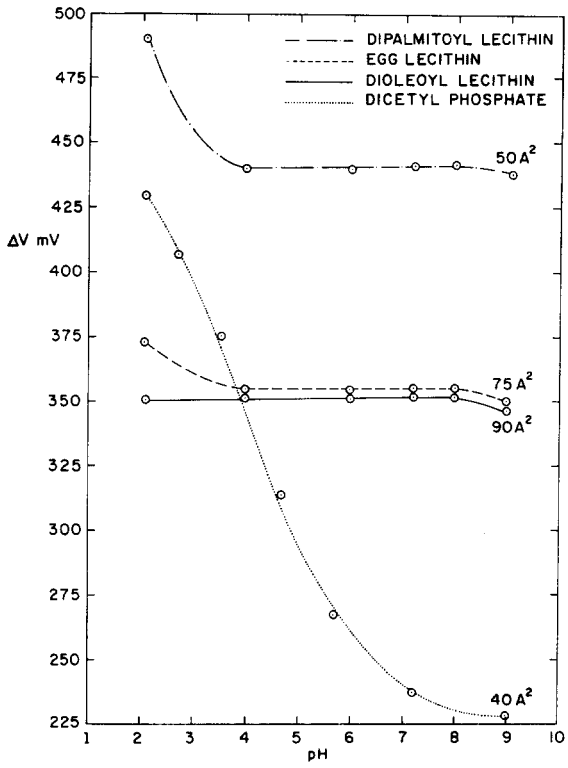

FIG. 3. The ΔV–pH curves of dicetyl phosphate and of dipalmitoyl, egg, and dioleoyl lecithins. The corresponding areas are selected at the surface pressure of 20 dynes/cm.

nonionic monolayers, the surface potential is not influenced by the pH of the subsolution, and consequently the ΔV–pH plot is a straight line with zero slope (8). Fig. 3 shows the ΔV–pH curves for monolayers of dicetyl phosphate and of dipalmitoyl, egg, and dioleoyl lecithin at areas/molecule of 40 A^2, 50 A^2, 75 A^2, and 90 A^2, respectively. These areas correspond to the same state of compression, namely a surface pressure of 20 dynes/cm. The curves in Fig. 3 show a striking difference between dicetyl phosphate and the lecithins, with respect to the neutralization of the phosphate group. Dicetyl phosphate shows a gradual ionization of the phosphate group as the pH of the subsolution increases. The zero slope of the dioleoyl lecithin curve in the pH range 2–8 indicates that these monolayers are effectively uncharged (nonionic) and suggests that the phosphate and trimethylammonium groups neutralize each other. In a smaller pH range, 4–8, dipalmitoyl and egg lecithins also showed the properties of uncharged monolayers. As the pH is decreased from 4 to 2, the extent of neutralization of the phosphate group as shown in Fig. 3 is in the order dipalmitoyl lecithin > egg lecithin > dioleoyl lecithin, which is the reverse of the order of their intermolecular spacing in monolayers (6). This suggests that neutralization of the phosphate group is related to intermolecular spacing in monolayers and in turn to unsaturation of fatty acyl chains of lecithin molecules.

Influence of Electrolyte Concentration of Subsolutions on Lecithin Monolayers

Surface Pressure–Area Curves. Surface pressure–area curves of dipalmitoyl, egg, or dioleoyl lecithin monolayers are essentially unaffected by the electrolyte concentration of the subsolution, although at high concentrations (10^{-1}M NaCl or $CaCl_2$), surface pressure–area curves indicated slight expansion of the monolayer (5 A^2/molecule).

Surface Potential–Electrolyte Concentration Curves. Surface potentials of lecithin monolayers are higher on subsolutions containing $CaCl_2$, because of the binding of Ca^{++} to phosphate groups in monolayers. ΔV–log C curves (analogous to the ΔV–pH curves) can be plotted for monolayers, where C is the electrolyte concentration of the subsolution (NaCl or $CaCl_2$). Fig. 4 shows the ΔV–log C curves for monolayers of dipalmitoyl, egg, and dioleoyl lecithins at areas/molecule of 50 A^2, 75 A^2, and 90 A^2, respectively. These areas correspond to the same state of compression, namely a surface pressure of 20 dynes/cm.

For charged monolayers, the slope of the ΔV–log C plot for a 10-fold change in electrolyte concentration of the subsolutions is related to the change in potential of the diffuse layer of ions (9, 10). But lecithin monolayers do not give rise to a diffuse layer of ions in the subsolution, since there is no net charge on the monolayers between pH

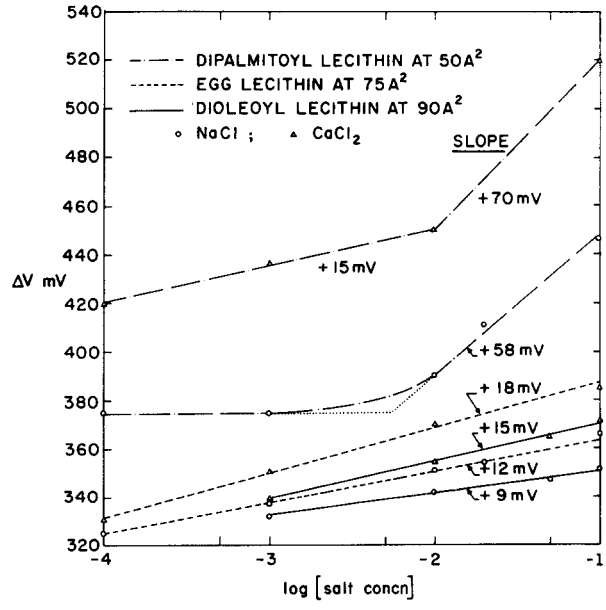

FIG. 4. The ΔV–log C curves of dipalmitoyl, egg, and dioleoyl lecithin monolayers for subsolutions of NaCl (O) and $CaCl_2$ (Δ). Values of the slopes represent the increase in surface potential upon a 10-fold increase in electrolyte concentration of the subsolution, pH 5.6 at 25 °C.

4 and 8 (see Fig. 3). Thus, the slope of the ΔV–log C plot in the case of lecithin does not indicate a change in the diffuse layer but shows the *interaction* of cations with lecithin molecules. In order to understand the ΔV–log C curves of lecithin monolayers, we should consider the effect of electrolyte concentration on the internal salt linkage of the lecithin molecule. When the salt concentration of the subsolution is increased, the cations compete for the anionic oxygen of the phosphate group with the trimethylammonium group of the lecithin molecule, with a consequent increase in the dissociation of the internal salt linkage between the phosphate and the trimethylammonium group. Therefore, the change in the surface potential per 10-fold increase in salt concentration is related to the binding constant of the phosphate group in lecithin monolayers. The theoretical treatment has not been developed to the extent that the absolute value of the binding constant can be calculated, but a comparison of the slopes of the ΔV–log C curves yields information about the relative strengths of the binding of cations.

The ΔV–log C plot for a nonionic monolayer is, of course, a straight line with zero slope (8, 11). On subsolutions of NaCl, the slopes (per order of magnitude of concentration) for monolayers of dioleoyl, egg, and dipalmitoyl lecithins are +9 mv, +12 mv, and +58 mv respectively; the corresponding slopes on subsolutions of $CaCl_2$ are +12 mv, +18 mv, and +70 mv. The higher values of the slope on subsolutions of $CaCl_2$ indicate that Ca^{++} is more effective than Na^+ in binding to the phosphate group of lecithins. For either Na^+ or Ca^{++}, the ability of lecithins to bind cations is in the order dipalmitoyl lecithin > egg lecithin > dioleoyl lecithin. Dipalmitoyl lecithin monolayers showed a peculiar behavior (Fig. 4), which can be explained as follows. Among the lecithins studied, dipalmitoyl lecithin monolayers have the smallest limiting area (44 A^2/molecule) and consequently the smallest intermolecular spacing between the phosphate groups. The negative and positive charges form two planes of high charge density, separated by two methylene groups (Fig. 2B). The binding of Na^+ to the phosphate group would be influenced by the repulsion due to the cationic charge in the plane of the trimethylammonium groups. Thus, the phosphate group is unable to bind Na^+ at low salt concentration. At concentrations higher than 0.005 M NaCl, there are enough Na^+ and Cl^- ions to diminish the effect of the cationic plane and the binding of Na^+ takes place (Fig. 4). A similar effect is also observed for subsolutions containing $CaCl_2$. Up to 0.01 M $CaCl_2$, the slope is +15 mv; above this concentration the slope is +70 mv because of the binding of Ca^{++} to adjacent phosphate groups. Egg and dioleoyl lecithins do not form cationic planes of such high charge density since the intermolecular spacing in their monolayers is larger (2, 6). Recently Bangham and Papahadjopoulos (12) have reported similar ΔV–pH and ΔV–log C plots for phosphatidyl serine monolayers.

Binding of Ca^{++} to Egg Lecithin and Egg Phosphatidic Acid Monolayers

Fig. 5 shows the surface potentials of egg lecithin and egg phosphatidic acid monolayers on subsolutions containing 0.02 M NaCl and 0.01 M $CaCl_2$ at pH 5.6 and 25°C. The phosphatidic acid monolayer showed four times more increase in surface potential when $CaCl_2$ was substituted for NaCl than did the egg lecithin monolayers. This again supports the conclusion that the trimethylammonium group partially prevents the binding of Ca^{++} to the phosphate group in egg lecithin monolayers (Fig. 2 B).

Orientation of Phosphoryl Choline Group and the Position of Ca^{++} in Lecithin Monolayers

In the above considerations, the phosphate and trimethylammonium groups are approximately parallel to the fatty acyl chains and perpendicular to the interface. On the other hand, a model has been proposed on theoretical grounds by Pethica (13) and also suggested by others (14, 15) in which the phosphate and trimethylammonium groups are coplanar, the plane being perpendicular to the fatty acyl chains of the lecithin molecule. This model seems unlikely, because (*a*) monolayers with

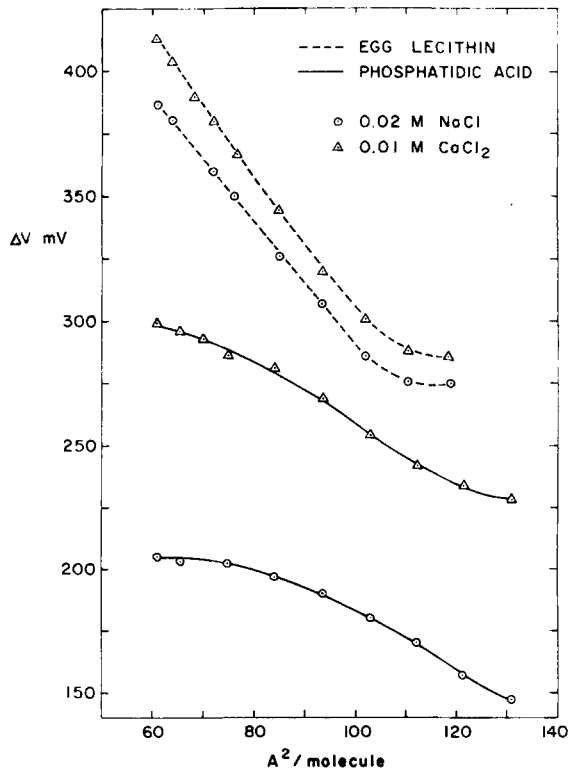

FIG. 5. Surface potential–area curves of egg lecithin and egg phosphatidic acid on subsolutions of 0.02 M NaCl (○) and 0.01 M $CaCl_2$ (△), pH 5.6 at 25°C.

such a structure should resemble the mixed (1:1) monolayers of dicetyl phosphate–eicosanyl trimethylammonium, which do not bind Ca^{++} (Fig. 1), whereas dipalmitoyl or distearoyl lecithins bind Ca^{++} strongly (2, 7); (*b*) formation of salt linkages between coplanar phosphate and trimethylammonium charges causes condensed, solid monolayers (Fig. 1), whereas dipalmitoyl and natural lecithin form liquid expanded monolayers; (*c*) monolayers of lecithins with widely different composition are uncharged (Fig. 3). This is much more easily explained by *internal* neutralization of ionic charges than by neutralization by oppositely charged groups of adjacent molecules, as the Pethica model proposes, for the intermolecular distances are very dependent on fatty acid composition; and (*d*) it is known from solubility data for alkyl phosphate and alkyl trimethylammonium (16, 17) that both these groups are strongly hydrophilic and therefore tend to dissolve in the subsolution. This alone would tend to pull the phosphoryl choline group out of any alignment parallel to the interface. The approximately vertical arrangement of the phosphoryl choline group, together with the formation of internal salt linkage, can account for the binding of Na^+ and Ca^{++}, the influence of pH, of unsaturation, and of cholesterol on lecithin monolayers.

The results reported here indicate that the internal salt linkage between phosphate and trimethylammonium groups is strengthened by increasing unsaturation of the fatty acyl chains (because of the corresponding increase in the intermolecular spacing) and also by the presence of cholesterol in monolayers of egg lecithin. This salt linkage is dissociated upon increasing the electrolyte concentration of the subsolution, Ca^{++} being more effective than Na^+ in dissociating this linkage.

As shown in Fig. 6, there are two positions available to Ca^{++} in lecithin monolayers. The upper position of Ca^{++} is more favorable than the lower because of the repulsion from the trimethylammonium groups. The Ca^{++} in the upper position should form an ionic dipole ($Ca^{++} \leftrightarrow O^-$) with the upper pole positive and the lower pole negative. This would increase the resultant vertical component of the lecithin dipole and hence the surface potential. Conversely, the lower position of Ca^{++} should decrease the surface potential. Surface potential measurements on lecithin monolayers showed an increase in surface potential in the presence of $CaCl_2$ in the subsolution (Fig. 4), which suggests that Ca^{++} is situated above the ionized oxygen in lecithin monolayers (Fig. 6). This is also in agreement with the electrostatic consideration discussed above.

We have shown (Fig. 4; reference 2) that the interaction of Ca^{++} with lecithin decreases with increasing unsaturation of fatty acyl chains and hence with intermolecular spacing in monolayers. The intermolecular spacing in a monolayer can be approximately calculated by assuming the "limiting area" as the area of a circle with radius *r;* then the intermolecular spacing is $2r$. Considering the limiting areas 44 A^2, 62 A^2, and 72 A^2 for dipalmitoyl, egg, and dioleoyl lecithins, respectively, the corresponding intermolecular spacings are 7.5 A, 8.88 A, and 9.58 A. These values lead to the conclusion that a change of only 1–1.5 A in the intermolecular spacing strikingly influences the interaction of lecithin with metal ions.

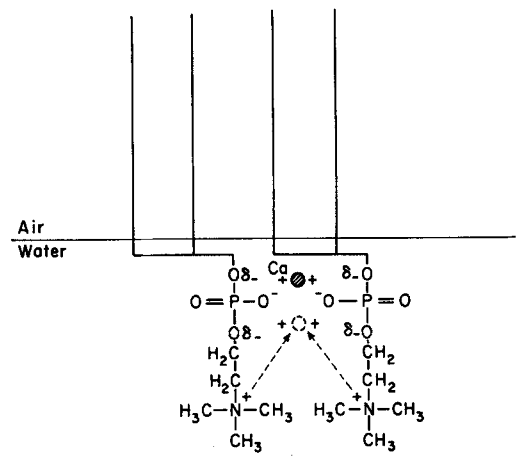

Fig. 6. The position of Ca^{++} in the lecithin monolayer. The arrow indicates repulsion of cationic trimethylammonium groups. The partial charge on the oxygen atoms is denoted by δ_-.

These results regarding the binding of Ca^{++} to lecithin monolayers as measured by the increase in surface potential are in contrast to ^{45}Ca adsorption (surface radioactivity) studies of Rojas and Tobias (18), who showed that ^{45}Ca does not bind to lecithin monolayers and desorbs from the monolayers at pH values greater than 5. This discrepancy is accounted for as follows. It is justifiable to assume that the excess of radioactivity of metal ions at the interface, after the spreading of the monolayers of alkyl sulfates, alkyl phosphates, or fatty acids, is due to the adsorption of metal ions at the interface. However, in the case of lecithin, two oppositely charged groups are involved, the phosphate group attracting $^{45}Ca^{++}$ ions, the trimethylammonium group repelling them. The net increase in radioactivity will depend upon the relative influence of these groups on Ca^{++} ions in the surface region. The apparent desorption of ^{45}Ca reported by Rojas and Tobias (18) suggests that the effect of the trimethylammonium is predominant over that of the phosphate group. This is expected since one calcium ion can be shared by a maximum of two adjacent phosphate groups, whereas two trimethylammonium groups could repel more than one calcium ion from the surface region and thus give rise to a decrease in surface radioactivity. It is stressed that the surface radioactivity is related to the

total number of metal ions present in the surface region and not to the *specific interaction* of metal ions with the charged groups in the monolayer. On the other hand, surface potential measurements are related to the *specific interaction* of metal ions with the charged groups in the monolayers, and not to the total number of metal ions present in the surface region. Thus, the surface potential is a more suitable approach to the study of the *interaction* of metal ions with phospholipid monolayers.

The ionic properties of aqueous dispersions of lecithins have been studied by potentiometric titration (19, 20), microelectrophoresis (21, 22), conductivity, and flocculation methods (23). The results presented here are derived exclusively from monolayer systems, and may or may not be applicable to studies on phospholipids dispersed in bulk solution, where the parameters related to intermolecular spacing and state of compression are not as well defined as in monolayers.

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