BBA 75178

SURFACE PROPERTIES OF ACIDIC PHOSPHOLIPIDS: INTERACTION OF MONOLAYERS AND HYDRATED LIQUID CRYSTALS WITH UNI- AND BI-VALENT METAL IONS

DEMETRIOS PAPAHADJOPOULOS*

A.R.C. Institute of Animal Physiology, Babraham, Cambridge (Great Britain) and Department of Biochemistry, State University of New York at Buffalo, Buffalo, N.Y. (U.S.A.)

(Received April 29th, 1968)

SUMMARY

The interaction of phospholipids with bivalent metals was studied by the use of monolayers and liquid crystals composed of purified, naturally occurring phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid and phosphatidylinositol. The properties under investigation were surface pressure, surface potential and ζ potential. Changes in these parameters were followed as a function of pH and bivalent metal ion concentration.

It was found that the acidic phospholipids interact strongly with bivalent metals at low concentrations (10^{-4}–10^{-3} M), in the presence of physiological concentrations of univalent salts (10^{-1} M). The interaction is accompanied by an increase in surface potential and a decrease in surface pressure (condensation) of monomolecular films. Of the phospholipids examined, phosphatidylserine and phosphatidic acid at pH values higher than 7.0, both possessing two negative groups per molecule, exhibited the highest affinity for bivalent metals.

On the basis of the results presented, a model was proposed for the complex of the latter two phospholipids with Ca^{2+}. The model involves coordination bonds between each Ca^{2+} and four phospholipid molecules resulting in a linear polymeric arrangement. The implications of the complex were discussed in terms of the physiological role of bivalent metals on membranes.

INTRODUCTION

The importance of phospholipids as structural and functional components of biological membranes has stimulated numerous studies on the surface properties of the purified compounds. Such investigations have included the use of phospholipids in the form of monomolecular films or as aqueous dispersions of liquid-crystalline particles[1,2]. These systems, composed of purified phospholipids, have served as

* Established Investigator of the American Heart Association. Present address: Department of Biochemistry, State University of New York at Buffalo, Buffalo, N. Y., U.S.A.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphatidylinositol; M, metal ions.

simplified models for biological membrane structure[3] although the orientation of the phospholipid molecules within the biological membrane may be considerably modified by binding to specific proteins[4].

The use of phospholipid liquid crystals as a model system for permeability studies has recently been described by BANGHAM, STANDISH AND WATKINS[5]. The system was based on the finding that egg yolk phosphatidylcholine, when dispersed in aqueous salt solution, formed "closed" multilamellar structures of limited permeability to K^+ trapped between the lamellae. Subsequently, BANGHAM and his coworkers[6,7] studied the permeability changes produced by general and local anaesthetics and the osmotic properties of the liquid-crystalline particles[8]. PAPAHADJOPOULOS AND MILLER[9] extended these studies to include a number of naturally occurring phospholipids. They investigated the effect of various physical and chemical parameters and described the formation of small unilamellar vesicles produced by ultrasonication. PAPAHADJOPOULOS AND WATKINS[10] employed the same liquid-crystalline systems to correlate gross morphology and chemical structure to the permeability characteristics exhibited by the various particles. In a preliminary study of the permeability properties of phosphatidylserine, PAPAHADJOPOULOS AND BANGHAM[11] reported on the effect of bivalent metals, temperature and local anaesthetics on the diffusion of K^+ and Na^+. The affinity of phospholipids in the form of sonicated suspension for various ions has been extensively studied by ABRAMSON *et al.*[12,13] and also by HENDRICKSON AND FULLINGTON[14] who reported on the stability constants between acidic phospholipids and bivalent metals.

The properties of phospholipid monolayers have been under intensive study by various groups of investigators for a considerable period of time. The more recent studies involving the use of synthetic phospholipids or well purified natural products have contributed appreciably to the available knowledge on molecular packing and orientation of these compounds at interfaces[15–18]. The interaction of phospholipids with Ca^{2+} has also been studied by the use of the binding of $^{45}Ca^{2+}$ on monolayers[19–21].

BANGHAM AND PAPAHADJOPOULOS[22] have recently reported on the effect of bivalent metals on the surface potential and surface pressure of phosphatidylserine monolayers. The work reported here is an extension of the above study, employing several naturally occurring phospholipids, with emphasis on the interaction of acidic phospholipids with bivalent metals. An attempt has been made to correlate the surface characteristics of monolayers with the behavior of hydrated liquid crystals obtained from the same compounds. This investigation was carried out in conjunction with a study of the permeability properties of the hydrated phospholipid liquid crystals[10] in an effort to relate physicochemical characteristics to biological membrane function.

MATERIALS AND METHODS

Materials

All reagents used were of analytical grade or of the highest purity available. The inorganic salts were heated at 500° for 4 h to eliminate trace organic materials. The water was double-glass-distilled over $KMnO_4$. The phospholipids used in this study were purified and characterized by methods described in detail elsewhere[9]. Phosphatidylserine (PS) and phosphatidylinositol (PI) were obtained from beef

brain. Phosphatidylcholine (PC), phosphatidylethanolamine (PE) from egg yolk and phosphatidic acid (PA) by enzymatic degradation of phosphatidylcholine.

Methods

The preparation of liquid crystals and the method of measuring diffusion rates of K^+, Na^+ and Cl^- have been described before[10].

Surface potential (ΔV) and surface pressure (π) were measured as already described[22] in a small (100 ml volume) polythene trough, the surface of which was separated into two compartments. One compartment was used for confining the film (21 cm^2), and the ionizing air-electrode (Americium-241 foil, suspended approx. 5 mm from the surface). The other compartment was used for inserting a glass electrode, a calomel electrode, an electrically driven stirrer and for the addition of reagents. A high-impedance electrometer (Vibron, Model 33B, Electr. Instr. Ltd.) was used with the ionizing air-electrode and the calomel electrode for surface potential measurements. The accuracy of the system was $\pm$ 0.5 mV. A sensitive bridge-circuit balance (Combustion Instr. Ltd.) was used for the measurement of the film pressure. A platinum wire (0.2 mm diameter) substituted for the conventional Wilhelmy vertical plate. The dipping end of the wire was replatinized frequently for better stability and was cleaned by flaming before each experiment. The accuracy of the system with the stirrer "on" was $\pm$ 0.5 dynes/cm.

The entire assembly was earthed and enclosed in a screened metal box. Monolayers were applied on the surface using a 2 mM solution of the phospholipid in light petroleum (b.p. 60–80°). For acid–base titrations 1 M or 5 M solutions of HCl, KOH, NaOH, or LiOH were added to a bulk-phase containing either KCl, NaCl or LiCl at a concentration of 145 mM unless otherwise specified. Bivalent metals (1 M solutions) were added to a bulk-phase containing 130 mM KCl, NaCl or LiCl and 14.5 mM Tris–HCl at pH 7.4 unless otherwise specified. Changes of ΔV, π, and pH were recorded simultaneously and continuously with a three-channel recorder.

Measurements of film pressure at different areas per molecule were measured in a conventional Langmuir trough assembly (1000 ml capacity) with a mica floating boom[23]. Because it was found difficult to maintain a film at high pressures with conventionally waxed glass barriers[23], the entire area containing the monolayer was surrounded by a polythene band. The band was made stationary by waxing it at appropriate positions on the two sides of the trough. The film was compressed with a barrier pushed from the clean side of the trough with a system of pullies taking up the slack of the excess polythene band.

Electrokinetic potential (ζ) measurements on phospholipid dispersions were performed by microelectrophoresis[24]. The electrophoretic mobility of the particles, in 145 mM salt solutions was converted to ζ-potential as described by DAVIES AND RIDEAL[23].

RESULTS AND DISCUSSION

General characteristics of phospholipid monolayers

The system of monolayer titration under constant area per molecule as described by BANGHAM AND PAPAHADJOPOULOS[22] is a relatively simple and quick method for identification of titratable groups. When used with naturally occurring phospho-

lipids, it was noticed that both ΔV and pressure (π) of the applied film suffered a decay with time in a linear fashion. The extent of the drop in positive ΔV and π depended on a number of parameters, *i.e.*, the type of ionizing air-electrode and its distance from the film, the unsaturation of the hydrocarbon chains of the film material, and the presence of air O_2. It would appear that the phenomenon is related to autoxidative changes initiated or promoted by the presence of ionizing radiation. Using a solid film of stearic acid and a Polonium point-radiation source, it was observed that areas in the immediate vicinity of the electrode registered lower ΔV while the rest of the film still gave the original ΔV value. Thus, by moving the electrode along a line, areas previously exposed to the electrode could be identified. The same phenomenon could be observed with a film of PS made solid (talcum test) by introduction of UO_2^{2+} in the bulk-phase. The use of the Americium foil as an ionization source, held at a distance of approx. 10 mm, minimized this effect. Thus, no loss of ΔV or π could be observed with films of stearic acid or hydrogenated egg lecithin. Nevertheless, films of natural phospholipids still showed a decrease of ΔV of approx. 0.5 mV/min and 0.1–0.2 dyne/cm per min in pressure. The loss was largely eliminated by removing oxygen from the system. This was accomplished by initially flushing the closed cabinet with N_2 and keeping a positive N_2 pressure during the experiment. N_2 was also bubbled through the salt solution on which the film was to be applied. No difference in the overall values for ΔV or π was observed between experiments performed in the presence of N_2 or in the presence of air.

A representative example of the variation of film pressure (π) and surface potential (ΔV) with area per molecule is given in Fig. 1. The phospholipid used in this case was PS. Curve I represents the increase in π as a film of PS on KCl (130 mM)–Tris–HCl (15 mM, pH 7.4) was compressed on the conventional Langmuir trough. A maximum (collapse) pressure of 48 dyne/cm was reached at an area approx. 50 Å^2 per molecule. Curve II was obtained when sequential amounts of PS were added and the film pressure was reached after each addition by equilibration rather than com-

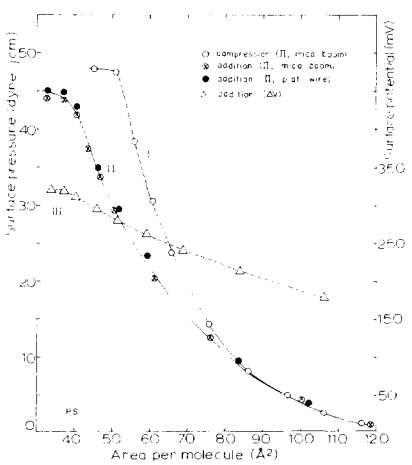

Fig. 1. Surface pressure–area and surface potential–area curves of phosphatidylserine monolayers on NaCl–Tris–HCl (pH 7.4). π/A curve obtained: by compression of PS on a Langmuir trough (○); by addition of consecutive amounts of PS on a Langmuir trough (⊗), or the small circular trough with platinum wire and microforce balance (●). $\Delta V/A$ curve obtained by addition of consecutive amounts of PS (△).

pression. It can be seen that approx. 20% more material was needed to reach the maximum (equilibrium) pressure, of 45 dyne/cm. Identical results were obtained using either the big trough with the floating "boom" or the small (21 cm^2) trough with the hanging platinum wire. Curve III represents the increase of ΔV during the addition of PS. Here the increase is almost linear with a plateau reached at the same area per molecule as in Curve II. Similar results can be obtained with films of the other phospholipids on monovalent salt solutions.

Titration of phospholipid films. Changes of ΔV with pH

Fig. 2 is a compilation of representative data for a variety of phospholipids depicting changes in ΔV with pH. The films were obtained by adding enough material on the surface to reach the equilibrium pressure. Some experiments were performed at pressures below the equilibrium with qualitatively similar results. The bulk-phase was 145 mM in monovalent salts. HCl or the appropriate base was added under the film during the experiment, while the bulk-phase was stirred continuously. Small differences between Na^+, K^+, and Li^+ were not significantly far from the experimental error to merit evaluation. Each curve represented in Fig. 2 is an average of 3–5 different experiments. The films were usually layered at the natural pH of the salt solution (approx. 5.5–6.0) with acid or base added subsequently. Most titrations were reversible, except for PA between pH 6.5 to 9.5, and for some preparations of PE between pH 6 to 10. All phospholipids indicated the presence of an ionizable group at pH below 3.5 which is probably the primary phosphate. PS possessed additional titratable groups between pH 3 and 5.5 (presumably the carboxyl) and above 9.2 (primary amino group). PA possessed an additional ionizable group between pH 6.5 and 9.5 which is probably the secondary phosphate. The primary amino group of PE was titratable at pH values above 6.5 with an accompanying big change in ΔV.

An additional group was observed with some preparations of PE, as revealed by a discontinuity in the descending curve shown in Fig. 2 between 5.5 and 7.5. This behavior could be due to some impurity, most likely either free fatty acid or phosphatidic acid, both deriving from hydrolytic degradation of the PE molecule. Since the ionization of the secondary phosphate in the PA film gives a total of only 25 mV

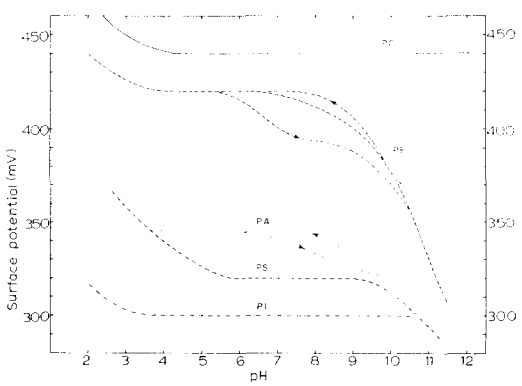

Fig. 2. Surface potential–pH curves obtained by titration of monolayers of different phospholipids at fixed area per molecule in circular trough. ———, PC; - - - - - -, PE; ·····, PA; ·· ·· ·· ··, PS; ·· ·· ·· ·· ·· ··, PI. Bulk-phase contained NaCl (145 mM).

decrease in ΔV, it could not contribute 30 mV as an impurity in the PE sample. On the other hand, when a film composed of a mixture of PC with 5 % stearic acid on 145 mM KCl was titrated with KOH, a similar decrease in ΔV (30 mV) was observed between 5.5 and 7.5. Further, when the solution was then back-titrated with acid, the 30-mV change in ΔV occurred between pH 9 and 8, a behavior similar to that observed with PE. Nevertheless, repeated extraction of these PE preparations in petroleum ether with 0.1 M bicarbonate solution did not eliminate this anomalous behavior. Finally, as shown in Fig. 2, films of PC and PI indicated no change in ΔV between pH 3.5 and 11.5. It should be noted here that exposure of the films at high pH for considerable length of time resulted in the appearance of ionizable groups (presumably hydrolytic products) titrating between pH 6 and 8. If lengthy exposure to extremes of pH were avoided, however, the curves were reversible upon back-titration.

Changes in π with pH

The ionization of a functional group was usually accompanied by an increase in film pressure provided that the starting pressure was below the maximum (equilibrium) pressure. Films of PS, and PA expanded as the pH increased and the π/pH curves closely followed the ΔV/pH curves (Fig. 3). If the films were at their equilibrium pressure at the pH below ionization, no expansion was recorded during the addition of alkali. However, when the pH was subsequently lowered by addition of acid, a decrease in pressure occurred during the protonation resulting in a film of lower pressure. This was an indication that some of the molecules had been "squeezed" out of the film during the expansion due to ionization and that the equilibrium between the excess material (possibly in the form of liquid-crystalline "rafts") and the molecules in the film was slow. When the films were titrated at pressures lower than the maximum equilibrium pressure (as shown in Fig. 3 for PA), the π changes were reversible. No change in pressure was observed over the entire pH range (2.5–11.5) for a film of PC.

Changes of π and ΔV with pH in the presence of Ca^{2+}

As shown in Fig. 3, addition of Ca^{2+} has a pronounced effect on the properties of PS monolayers. Curve 3–4 is a back-titration of a PS film (titrated as shown in Curve 1–2), after the addition of Ca^{2+} to a final concentration of 1 mM at pH 11.0. The immediate fall of film pressure by 19 dyne/cm (difference between Points 2 and 3) is characteristic of the condensation of PS films in the presence of Ca^{2+} as described earlier[22]. As shown by Curve 3–4, the film expands only slightly after addition of acid, indicating removal of some Ca^{2+} during the protonation of the functional groups. Nevertheless, Point 4 is still at a lower pressure than Point 1, indicating residual adsorption of Ca^{2+} on PS even at such a low pH. The addition of EDTA (in equimolar amounts to Ca^{2+}, in neutral or alkaline pH range) results in expansion of the film to the original pressure (Curve 1–2), indicating complete removal of Ca^{2+} from the surface.

Included in Fig. 3 (Curve PS/Ca^{2+}), is a titration of the same amount of PS as in Curve 1–2 but under conditions where the film is applied on a 145 mM KCl solution already containing 1 mM $CaCl_2$. In this case no change in film pressure could be recorded during repeated titration with either acid or alkali. The difference between

Curves PS/Ca^{2+} and PS + Ca^{2+} could be considered as an indication that Ca^{2+} may occupy more than one position in the PS monolayer. An experiment which illustrates this point further is presented in a following section.

Changes in ΔV during the titration of the phospholipids in the presence of 1 mM $CaCl_2$ (and 145 mM monovalent salts) are presented in Fig. 4. The PC curve is identical to that shown in Fig. 2, indicating no appreciable adsorption of Ca^{2+}. The same is true for PE below pH 7.0 although comparison with the PE curve in Fig. 2. indicates that some Ca^{2+} is bound at higher pH. The titration of PA in the presence of Ca^{2+} gives the "mirror image" curve of the one shown in Fig. 2. The increase in ΔV due to 1 mM Ca^{2+} is 20 mV between pH 3 and 7, and 65 mV above pH 9.0. The effect of Ca^{2+} can also be seen as a shift of the apparent pK_2 of PA to lower pH, from 8.1 to 7.1 (mid-points of the two ΔV/pH curves for PA, Figs. 2 and 4).

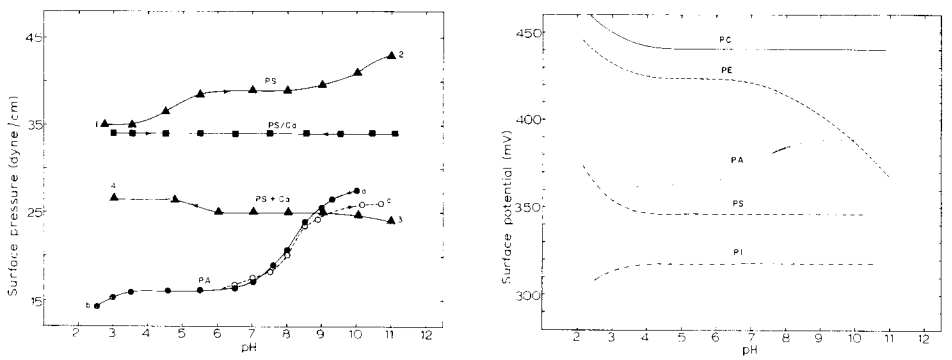

Fig. 3. Surface pressure–pH curves obtained by titration of PS and PA films on NaCl (145 mM). Titration of PS (▲) Points 1 to 2; addition of $CaCl_2$ (1 mM final concn.) at Point 2 produces contraction to Point 3; back-titration of the same film after addition of $CaCl_2$ (▲) Points 3 and 4. Titration of a PS film layered on NaCl (145 mM)–$CaCl_2$ (1 mM) (■). Titration of a PA film on NaCl (143 mM)–Tris–HCl (2 mM) (● or ○). Addition of acid, Points a to b; addition of alkali, Points b to c.

Fig. 4. Surface potential–pH curves obtained by titration of monolayers of different phospholipids at fixed area per molecule in circular trough, in the presence of Ca^{2+}. ——, PC; ------, PE; ·····, PA; - - - - - -, PS; - · - · - · - · - · -, PI. Bulk-phase contained NaCl (145 mM) and $CaCl_2$ (1 mM).

Similar behavior was observed with monolayers of PI. The pertinent curve, also shown in Fig. 4, demonstrates an increase of approx. 20 mV at low pH and a plateau above pH 4.0

The effect of Ca^{2+} on the titration curve of PS was quite different from its effects on the curves of PA and PI. As shown in Fig. 4, ionization of the phosphate group was indicated by a decrease in ΔV at low pH followed by a plateau above pH 4.0, with no further change in ΔV during the titration of the carboxyl and amino groups. This could be interpreted as indication for multiple binding of Ca^{2+}, involving the carboxyl and the de-protonated amine as well as the phosphate. A model of the proposed complex will be discussed later.

Changes in ΔV and π with bivalent metals

Titrations with bivalent metals were performed in the presence of 130 mM KCl or NaCl. The pH was usually adjusted to 7.4 with Tris–HCl (15 mM) unless otherwise

indicated, keeping the total monovalent salt concentration at 145 mM. The films were layered in the absence of divalent metals by adding enough material on the surface to reach a pressure near equilibrium, usually 43–44 dyne/cm. Changes in film pressure (π) and surface potential (ΔV) were recorded simultaneously. Addition of bivalent metals under films of the neutral phospholipids (PC, and PE at pH less than 7.0) had very little effect on either π or ΔV, indicating no appreciable interaction. On the other hand, there was substantial interaction with films of acidic phospholipids as indicated by an increase in ΔV and a decrease in π. The effect of various bivalent metals on the ΔV and π of films of PS, PA, and PI is shown in Figs. 5 and 6. In general the main characteristics of these titrations were: (a) a condensation of the film as indicated by the decrease of film pressure starting at very low bivalent metal concentrations and

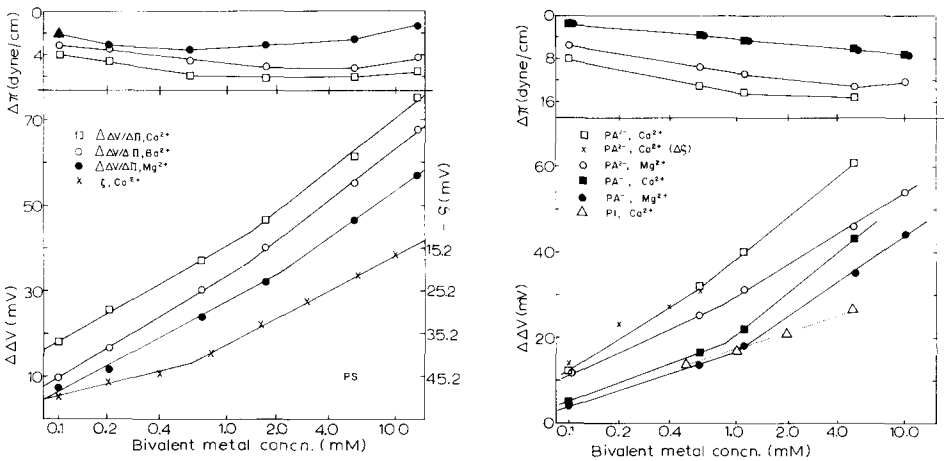

Fig. 5. Effect of bulk-phase concentration of bivalent metals on surface potential (ΔV) and surface pressure (π) of phosphatidylserine monolayers. The bulk-phase contained NaCl (130 mM)–Tris–HCl (15 mM) (pH 7.4). $\Delta\Delta V$ and $\Delta\pi$ during the addition of: $CaCl_2$ (□), $BaCl_2$ (○), $MgCl_2$ (●). Included in same figure: changes in ζ of PS liquid crystals in NaCl–Tris–HCl (pH 7.4), at different $CaCl_2$ concentrations (×). The value of ζ (−55.2 mV) corresponding to o $\Delta\Delta V$ is the ζ-potential of PS in NaCl–Tris without Ca^{2+}.

Fig. 6. Effect of bulk-phase concentration of bivalent metals on surface potential (ΔV) and surface pressure (π) of phosphatidic acid monolayers at two different pH's. The bulk-phase contained either NaCl (130 mM)–Tris–HCl (15 mM) (pH 7.6) or NaCl (145 mM) (pH 5.6). $\Delta\Delta V$ and $\Delta\pi$ during the addition of: $CaCl_2$ at pH 7.6 (□) and 5.6 (■), $MgCl_2$ at pH 7.6 (○) and 5.6 (●). Also included: $\Delta\Delta V$ of monolayers of PI during the addition of $CaCl_2$ (△); and changes in electrokinetic potential ($\Delta\zeta$ in mV) of PA liquid crystals in NaCl–Tris–HCl (pH 7.6) at different $CaCl_2$ concentrations (×). The ζ of PA liquid crystals before the addition of $CaCl_2$ was −64 mV.

reaching a minimum (different for each metal and each phospholipid) at concentrations between 1 mM and 10 mM; (b) an increase of ΔV in linear relationship to the log of the bivalent metal concentration (within a certain concentration range), with the slope becoming steeper at higher concentrations. A single slope was observed only in the case of the interaction of PI with Ca^{2+} (Fig. 6, triangles) and PS with Ca^{2+} at pH 3.0 (ref. 22). It is possible that the steep slope indicates the titration of two negative groups (carboxyl and phosphate for PS, primary and secondary phosphate for PA) while the titration of only one group (as in the case of PI, and PS at low pH) results in a shallower slope.

SHAH AND SCHULMAN[18] have interpreted the biphasic relation found with PC and Ca^{2+} as a competition of Ca^{2+} with the quaternary ammonium group of PC. In the present experiments the Ca^{2+} would be competing with the protons of the amino group of PS and the phosphate group of PA. This competition would favor Ca^{2+} as the concentration in the bulk-phase increases. In the case of PA, an increase in pH would reduce the competition, resulting in a steeper slope at lower concentrations of Ca^{2+}. This interpretation is supported by the data presented in Fig. 6. It is conceivable that the change of slope discussed above indicates a transition within the phospholipid–M^{2+} complex from a simple dimer or trimer to a polymer involving chelation of the metal (M), a possibility which will be dealt with in detail later. The significance of the change in slope of ΔV *vs.* log $[M]^{2+}$ curve is of interest because it occurs at approximately the same concentration at which permeability changes occur in hydrated liquid crystals[10,11]. The interaction of PS and PA with Ca^{2+} was also investigated by the method of microelectrophoresis. Fig. 5 depicts the changes in the electrokinetic potential ($\Delta\zeta$) of PS liquid crystals as the bulk-phase concentration of Ca^{2+} increases. The results confirm the biphasic relation found with the ΔV measurements, and indicate that the observed changes can be assigned to neutralization of the ionic dipoles rather than to differences in permanent dipole orientation. The $\Delta\zeta$ values for PA^{2-}, Ca^{2+} (shown in Fig. 6) closely follow the ΔV measurements at the same pH.

Other points of interest shown in Figs. 5 and 6 are the differences between Ca^{2+}, Ba^{2+}, Mg^{2+}. It is apparent that PS exhibits more discrimination than PA, but the relative affinity is the same for both phospholipids, *i.e.*, $Ca^{2+} > Ba^{2+} > Mg^{2+}$. It can also be seen in Fig. 6 that PA reacts more strongly with bivalent metals at pH 7.4 as compared to pH 6. This observation is corroborated by the effect of Ca^{2+} on the permeability of PA liquid crystals at two different pH values[25]. It has been found that the concentration of Ca^{2+} producing a permeability change to PA liquid crystals is 0.1 mM at pH 7.4, but 1.0 mM at pH 6.0.

Small differences in ΔV were also observed during titration of PS by Ca^{2+}, depending upon the monovalent cation present. In general, the $\Delta\Delta V$ values were higher in the presence of Na^+ compared to K^+, and higher with K^+ compared with Li^+. Due to the technical difficulties involved, it was not possible to quantitate the differences or to ascertain whether there were small differences in the slopes of the titration curves. It should be pointed out that permeability studies have shown significant differences in the efficacy of Ca^{2+} to increase the permeability of PS to $^{42}K^+$ depending upon the presence of K^+ or Na^+ (see ref. 11).

Compression of phospholipid monolayers

The condensation of a phospholipid film produced by 1 mM Ca^{2+} in the presence of 145 mM univalent salts, as described in the preceding section, is at variance with previous studies on the interaction of Ca^{2+} with PS (ref. 19). The experiments just described, however, involved the addition of Ca^{2+} under a pre-existing film rather than the more usual method of spreading the film on a Ca^{2+}-containing solution. In order to investigate any differences due to the method employed, the following experiments were performed in a conventional Langmuir trough with the film spread on a bulk-phase initially containing the indicated concentration of the particular ions in question. Special precautions as described in *Methods* were taken to avoid leakage at high film pressures.

Fig. 7A shows the relationship between film pressure and area per molecule for a film of PS on NaCl–Tris (145 mM, pH 8) with and without Ca^{2+} (1 mM). It can be seen that the PS film was slightly condensed in the presence of Ca^{2+}. The difference in film pressure at 52.5 $Å^2$ per molecule (which would be comparable to the packing during the titration experiments) was 5.0 dyne/cm, a value which was somewhat lower than the maximum condensation produced by 1 mM Ca^{2+} during titration of PS (6.0 dyne/cm, Fig. 5). The compression of a film of PC is also given in Fig. 7A for comparison. In agreement with previous studies[17,18] no condensation was observed with PC in the presence of Ca^{2+}. The collapse pressure of both PS and PC films was 47 dyne/cm and that of the PS–Ca^{2+} film, only 0.5 dyne/cm higher.

The condensation of PA by Ca^{2+} and Mg^{2+} at two different pH values is shown in Figs. 7B and 7C. Here the film reached collapse pressure at approx. 45 $Å^2$ per molecule (as compared to approx. 50 $Å^2$ per molecule for PS and PC), and this maximum pressure was significantly lower in the presence of bivalent metals. This was observed very clearly in the case of Ca^{2+} at pH 8.0 (Fig. 7C, collapse pressure 33 dyne/cm) and was also noted with Ba^{2+}, not shown here. This collapse of the film at lower pressures would account for the 14–16 dyne/cm condensation observed during titration of PA with Ca^{2+} and Ba^{2+} at pH 7.6 (Fig. 6). It is of interest to note here that the PA–Ca^{2+} films could be compressed momentarily to pressures up to 45 dyne/cm at which time they behaved as solid films (talcum test), a behavior drastically different to that in the absence of Ca^{2+}. The significance of the apparent change in viscosity can be seen in connection with the proposed PA–Ca^{2+} complex.

The following experiment was designed to investigate more directly the differences in ΔV and π of PS films depending on whether the Ca^{2+} was added before or after PS. Fig. 8 indicates the values of ΔV and π for the same amount of PS layered on the same trough containing NaCl–Tris (145 mM, pH 8.0) without Ca^{2+} (solid line), and with Ca^{2+}, 0.7 mM (interrupted line). It can be seen that initially the Ca^{2+}–PS

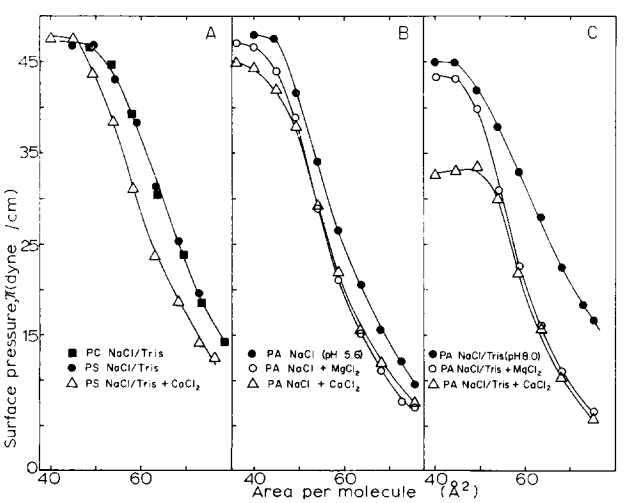

Fig. 7. Surface pressure–area curves of PS and PA monolayers with and without bivalent metals. A. π/A curves of PC (■) and PS (●) on NaCl–Tris–HCl (pH 7.4); PS on same bulk-phase containing $CaCl_2$, 1 mM (△). B. PA on NaCl, pH 5.6 (●); containing $MgCl_2$, 1 mM (○); or $CaCl_2$, 1 mM (△). C. PA on NaCl–Tris (pH 8.0) (●); containing $MgCl_2$, 1 mM (○); or $CaCl_2$, 1 mM (△).

film was only slightly condensed but the ΔV was 80 mV higher. Addition of 0.7 mM Ca^{2+} under the PS film (first arrow) decreased the film pressure by 6 dyne/cm (condensation) and increased the ΔV by 40 mV (a value comparable to the titration shown in Fig. 6). At this stage the two films are identical in terms of amounts of Ca^{2+} in bulk-phase and PS on the surface, but differ significantly in π and ΔV. Addition of EDTA (1.0 mM final concentration) brings the two films to the same value of π and ΔV, and further addition of Ca^{2+} now has the same effect on both films. This evidence, although rather preliminary, indicates that there might be two positions for Ca^{2+} binding, depending on the method of presentation of the metal to the phospholipid.

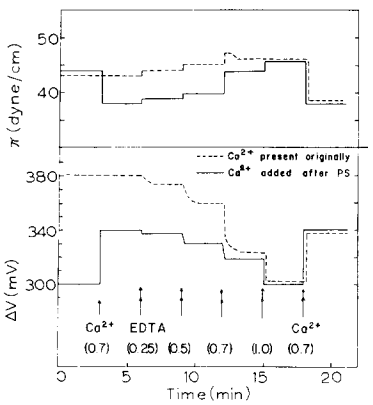

Fig. 8. Surface pressure (π) and surface potential (ΔV) of a film of PS on NaCl–Tris–HCl (pH 7.4) with $CaCl_2$: – – – –, PS film applied on a bulk-phase containing $CaCl_2$ (0.7 mM); ———, the same amount of PS applied on a bulk-phase containing only NaCl–Tris–HCl; subsequent addition of Ca^{2+} and EDTA is indicated by arrows. Concentrations are also indicated in parentheses.

Electrokinetic potential of phospholipid liquid crystals

As a corollary to the study of phospholipid–metal associations in monolayers just described, an investigation was undertaken on the effects of pH and bivalent metals on the ζ-potential of liquid-crystalline particles as measured by electrophoretic mobility. The effect of Ca^{2+} on the mobility of PS and PA has already been described in Figs. 5 and 6. It is evident that the presence of Ca^{2+} decreases the mobility as would be expected by a neutralization of the negative charges on the surface of the particles. It is of interest that the negative charge is retained even at relatively high concentration of Ca^{2+} (—16.8 mV at 10 mM for PS). This is rather unexpected in view of the finding that 1 equiv of Ca^{2+} (1/2 Ca^{2+}) is bound to the surface per PS molecule at 1 mM $CaCl_2$ concentration[22]. Since there is only one extra negative charge in each PS molecule, the presence of one equivalent of Ca^{2+} would be expected to neutralize completely the negative charge. It is possible that during the formation of the PS–Ca^{2+} complex the primary amino group loses its proton and the nitrogen participates in the complex with a co-ordinating bond to Ca^{2+}. Evidence for this has been presented earlier in this paper during the titration of a PS film in the presence of Ca^{2+} (Fig. 4). ABRAMSON, KATZMAN AND GREGOR[13] have reported on the release of H^+ from PS at neutral pH following the addition of Ca^{2+}. The only group that could release H^+ at this pH would be the primary amine. A PS–Ca^{2+} complex

implicating the amino group in intramolecular binding has been proposed recently by HENDRICKSON AND FULLINGTON[14]. The PS–Ca^{2+} complex proposed in the following section involves similar co-ordination bonds with the amino group but between vicinal PS molecules.

The effect of bulk-phase pH on the electrophoretic mobility of several pure phospholipids is shown in Fig. 9. The curves for PC and PE are characteristically similar to the ones obtained by the monolayer technique following changes in ΔV. The curve for PS indicates a significant deviation from the ΔV/pH curve of Fig. 2. Thus, there is no significant increase in mobility at high pH as would be expected from the ionization of the amino group. This could be taken as an indication that the

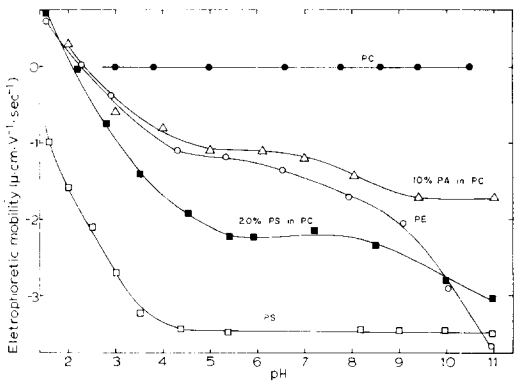

Fig. 9. Electrophoretic mobility–pH curves for various phospholipid liquid-crystalline particles. Bulk-phase contained NaCl (145 mM) and HCl or NaOH to bring the pH to desirable point. (●), PC; (○), PE; (△), 10 % PA in PC; (■), 20 % PS in PC; (□), PS. Mixed liquid crystals were obtained by mixing the two components in chloroform, evaporating to dryness and dispersing in NaCl.

amino group is located in the interior, away from the hydrodynamic slip-plane of the ζ-potential and consequently its ionization has a negligible influence on the electrophoretic mobility. However, when mixtures of less than 50 % PS in PC were electrophoresed at different pH's, the ionization of the amino group was quite apparent above pH 8.0 as shown in Fig. 9 for a 20 % PS in PC mixture. The same phenomenon was observed with PA where the ionization of the secondary phosphate group (10 % PA in PC, Fig. 9) was quite obvious in PA–PC mixtures, but not with pure PA. The conclusion to be drawn from these observations is that the microelectrophoresis method is not sensitive with highly charged surfaces such as those of the acidic phospholipids with more than one charge per 50–60 $Å^2$. The applicability of the equation relating surface potential with mobility to only moderately charged surfaces (about 30 mV) has been discussed by HAYDON[26].

The phospholipid–bivalent metal complex

Data presented in this paper emphasize several aspects of the interaction between phospholipids and metal ions. The affinity of acidic phospholipids for the different divalent metals and the ubiquitous presence of these compounds in biological membranes points out the relevance of such interactions to physiological problems.

The importance of bivalent metals on the structure and function of biological

membranes cannot be over-emphasized. Outstanding examples are the effect of Ca^{2+} on the permeability of red cells[27], epithelial cell junctions[28] and squid axon[29]. In addition, Ca^{2+} is a well known participant in mitochondrial function[30], cell contact phenomena and cell deformability[31], and activation of prothrombin[32]. TASAKI AND SINGER[33] have formulated a "macromolecular theory" for excitation phenomena, involving the binding of divalent metals on membrane negative sites. The molecular site for these events has not been discussed by these authors, but the acidic phospholipid–bivalent metal complex would be a reasonable possibility. Indeed, ABOOD[34] has recently elaborated on the possibilities of phosphate–Ca^{2+} complexes and their importance for membrane phenomena. Similarly, TOBIAS[35] has proposed a theory of excitation involving Ca^{2+} binding on PS and its replacement by K^+ during action potential.

Using stearic acid monolayers, DEAMER AND CORNWELL[36] have recently postulated a two-dimensional coordination complex involving Ca^{2+} and carboxyl groups. The same workers have pointed out that such a two-dimensional complex could not exist with phospholipids and Ca^{2+} because of the relatively large area occupied by the phospholipid molecules[36]. However, the shape of the individual phospholipid molecules, as derived from molecular models[37], indicates that molecules can be packed in such a way as to allow the phosphate group to occupy a position close to one of the small sides of a parallelogram. The dimensions of each side of the parallelogram would be determined by the conformation of the individual fatty acid chains. In this case the divalent metal can approach four phosphates close enough to form linear polymers of the type indicated diagramatically in Fig. 10 for PA. Similar coordination complexes have been proposed for Ca^{2+}–polyphosphates[38] and also for Ca^{2+}–ATP (see ref. 39). It is of interest that the stability constant of the Ca^{2+}–PA complex calculated by ABRAMSON *et al.*[12] is $1.6 \cdot 10^4$, a value similar to some of the reported constants for the Ca^{2+}–ATP complex[39,40]. The model shown in Fig. 10 involves only four of the coordination valencies of Ca^{2+}. The other two (octahedral arrangement) could be filled either by two molecules of water or by the remaining oxygens of the phosphate.

Fig. 10 also includes a diagramatic representation of a Ca^{2+}–PS complex involving all six coordinating bonds of Ca^{2+} which also results in a linear polymeric

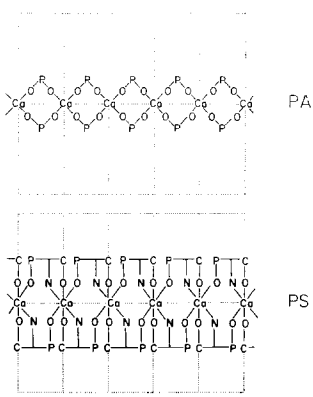

Fig. 10. Diagramatic representation of phosphatidic acid–Ca^{2+} and phosphatidylserine–Ca^{2+} complexes. Area outlined by dotted line represents the molecular area of one phospholipid molecule with the chains normal to the plane of the page.

arrangement. Molecular models of the complex have indicated no steric hindrance to such an arrangement. The increase in viscosity and decrease in hydration resulting from the above complexes could possibly explain the changes in permeability properties of liquid crystals observed earlier[10,11] and could account for some of the effects of bivalent metals on biological membranes. It should be stated here that the relevance of the proposed complexes to biological membranes is based on the assumption that natural membranes contain a coherent bilayer or monolayer of phospholipid molecules. Because of the lack of direct evidence for such an orientation, the existence of the proposed complexes within natural membranes should be considered only hypothetical.

There is some discrepancy in the published literature on the amount of bivalent metals bound by different phospholipids. In the presence of physiological concentration of monovalent salts, it has been estimated that 1 equiv of Ca^{2+} is bound per PS molecule at a bulk Ca^{2+} concentration of 1 mM (ref. 22). The complex proposed here is in agreement with this ratio. Under the same conditions PC does not bind bivalent metals to any appreciable extent. The studies by SHAH AND SCHULMAN[17,18] have shown that there is considerable affinity between dipalmitoyl PC and bivalent metals, but this is greatly diminished in the presence of unsaturated fatty acid chains. KIMIZUKA *et al.*[20] have found that $^{45}Ca^{2+}$ adsorbs to PC films but can be displaced by monovalent salts. ROJAS AND TOBIAS[19] have found that 1 Ca^{2+} (2 equiv) is bound per molecule of PS. Nevertheless, this measurement was made with PS monolayers and $^{45}Ca^{2+}$ on a bulk-phase of low I. When the concentration of KCl or NaCl was increased to 100 mM the ratio was found to be 0.17 Ca^{2+}/PS, with Ca^{2+} bulk concentration of 0.1 mM. This value is lower than the one found by BANGHAM AND PAPAHADJOPOULOS[22], but the discrepancy could be accounted for by the difference in Ca^{2+} concentration used in the two studies.

It can be concluded that at the physiological monovalent salt concentration only the acidic phospholipids bind strongly to bivalent metals. Among the acidic phospholipids, PS and PA (also the di- and tri-phosphoinositides not considered here) have the ability to complex with the divalent metals through coordination–chelation binding. It is conceivable that such complexes are involved in the physiological role of these phospholipids in biological membranes.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. A. D. BANGHAM for his guidance and support throughout this project, which was carried out in his laboratory at the Institute of Animal Physiology. I would also like to thank Mr. N. MILLER for his expert technical assistance. This work was performed during the tenure of an Advanced Research Fellowship of the American Heart Association (No. 65A29).

REFERENCES

1 A. D. BANGHAM, in D. KRITCHEVSKY AND R. PAOLETTI, *Advances in Lipid Research*, Vol. 1, Academic Press, New York, 1963, p. 65.
2 D. G. DERVICHIAN, in J. A. V. BUTLER AND H. E. HUXLEY, *Progress in Biophysics*, Vol. 14, Pergamon, Oxford, 1964, p. 265.
3 J. F. DANIELLI AND H. DAVSON, *J. Cellular Comp. Physiol.*, 5 (1935) 495.

4 L. L. M. VAN DEENEN, in R. T. HOLMAN, *Progress in Chemistry of Fats and Other Lipids*, Vol. 8, Pergamon, Oxford, 1965, p. 1.
5 A. D. BANGHAM, M. M. STANDISH AND J. C. WATKINS, *J. Mol. Biol.*, 13 (1965) 238.
6 A. D. BANGHAM, M. M. STANDISH AND N. MILLER, *Nature*, 208 (1965) 1295.
7 P. B. BENNET, D. PAPAHADJOPOULOS AND A. D. BANGHAM, *Life Sci.*, 6 (1967) 2527.
8 A. D. BANGHAM, J. DE GIER AND G. D. GREVILLE, *Chem. Phys. Lipids*, 1 (1967) 225.
9 D. PAPAHADJOPOULOS AND N. MILLER, *Biochim. Biophys. Acta*, 135 (1967) 624.
10 D. PAPAHADJOPOULOS AND J. C. WATKINS, *Biochim. Biophys. Acta*, 135 (1967) 639.
11 D. PAPAHADJOPOULOS AND A. D. BANGHAM, *Biochim. Biophys. Acta*, 126 (1966) 185.
12 M. B. ABRAMSON, R. KATZMAN, H. GREGOR AND R. CURCI, *Biochemistry*, 5 (1966) 2207.
13 M. B. ABRAMSON, R. KATZMAN AND H. P. GREGOR, *J. Biol. Chem.*, 239 (1964) 70.
14 H. S. HENDRICKSON AND J. G. FULLINGTON, *Biochemistry*, 4 (1965) 1599.
15 L. L. VAN DEENEN, U. M. T. HOUTSMULLER, G. H. DE HAAS AND E. MULDER, *J. Pharm. Pharmacol.*, 14 (1962) 429.
16 P. J. ANDERSON AND B. A. PETHICA, *Proc. Intern. Conf. Problems Lipids, 2nd, Ghent, 1955*, Butterworths, London, 1956, p. 24.
17 D. O. SHAH AND J. H. SCHULMAN, *J. Lipid Res.*, 6 (1965) 341.
18 D. O. SHAH AND J. H. SCHULMAN, *J. Lipid Res.*, 8 (1967) 227.
19 E. ROJAS AND J. M. TOBIAS, *Biochim. Biophys. Acta*, 94 (1965) 394.
20 H. KIMIZUKA, T. NAKAHARA, H. VEJO AND A. YAMAUCHI, *Biochim. Biophys. Acta*, 137 (1967) 549.
21 H. HAUSER AND R. M. C. DAWSON, *European J. Biochem.*, 1 (1967) 61.
22 A. D. BANGHAM AND D. PAPAHADJOPOULOS, *Biochim. Biophys. Acta*, 126 (1966) 181.
23 J. T. DAVIES AND E. K. RIDEAL, *Interfacial Phenomena*, Academic Press, New York, 1963, p. 134.
24 A. D. BANGHAM, R. FLEMANS, D. H. HEARD AND G. V. F. SEAMAN, *Nature*, 182 (1958) 642.
25 D. PAPAHADJOPOULOS, in preparation.
26 D. HAYDON, in J. F. DANIELLI, K. G. A. PANKHURST AND A. C. RIDDIFORD, *Recent Progress in Surface Science*, Vol. I, Academic Press. New York–London, 1964 p. 94.
27 J. F. HOFFMAN, *Circulation*, 26 (1962) 1201.
28 W. R. LOEWENSTEIN, *J. Colloid Sci.*, 25 (1967) 34.
29 B. FRANKENHAEUSER AND A. L. HODGKIN, *J. Physiol. London*, 137 (1957) 218.
30 A. L. LEHNINGER, *Ann. N. Y. Acad. Sci.*, 137 (1966) 700.
31 L. WEISS, *J. Cell Biol.*, 35 (1967) 347.
32 D. PAPAHADJOPOULOS AND D. J. HANAHAN, *Biochim. Biophys. Acta*, 90 (1964) 436.
33 I. TASAKI AND I. SINGER, *Ann. N. Y. Acad. Sci.*, 137 (1966) 792.
34 L. G. ABOOD, *Intern. Rev. Neurol.*, 9 (1966) 223.
35 J. M. TOBIAS, *Nature*, 203 (1964) 13.
36 D. W. DEAMER AND D. G. CORNWELL, *Biochim. Biophys. Acta*, 116 (1966) 555.
37 F. A. VANDENHEUVEL, *J. Am. Oil Chemist's Soc.*, 42 (1965) 490.
38 J. R. VAN WAZER AND D. A. CAMPANELLA, *J. Am. Chem. Soc.*, 72 (1950) 655.
39 A. E. MARTEL AND G. SCHWARZENBACH, *Helv. Chim. Acta*, 39 (1956) 653.
40 V. DISTEFANO AND W. F. NEUMAN, *J. Biol. Chem.*, 200 (1953) 759.