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Polar-Group Behaviour in Mixed Monolayers of Phospholipids and Fusogenic Lipids

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1. The surface potentials of mixed monolayers of synthetic phospholipids with lipids that are fusogenic for hen erythrocytes were investigated. 2. At pH5.6 and 10, but not at pH2, mixed monolayers of the fusogenic lipid, glycerol mono-oleate, with phosphatidylcholine exhibited negative deviations from the ideality rule in surface potential per molecule which were accompanied by negative deviations in mean molecular area. 3. Interactions of this type were not seen with chemically related but non-fusogenic lipids, nor were they found in mixed monolayers of any of the lipids with phosphatidylethanolamine. 4. Experiments with dihexadecyl phosphate and hexadecyltrimethylammonium indicated that the complete head group of phosphatidylcholine is required for its observed behaviour with fusogenic lipids. 5. Bivalent cations (Ca^{2+}, UO_2^{2+} or Zn^{2+}) in the subphase at pH5.6 significantly modified the behaviour of mixed monolayers of fusogenic lipids with phospholipids; there was a parallel perturbing effect of fusogenic lipids on interactions between monolayers of phospholipids and bivalent cations. 6. Possible molecular interactions of fusogenic lipids with membrane phospholipids, and the role of Ca^{2+}, are discussed which may be relevant to cell fusion in erythrocytes induced by low-melting lipids in the presence of Ca^{2+}.

Lipids that are able to induce hen erythrocytes to fuse into multinucleated cells (Ahkong *et al.*, 1973) show a characteristic behaviour in mixed monolayers with several species of phospholipids at the air/water interface, which was reported previously (Maggio & Lucy, 1975). Fusogenic lipids were found to exhibit negative deviations from ideality, in plots of mean molecular area against composition, when mixed in monolayers with erythrocyte lipids, natural and synthetic preparations of phosphatidylcholine, sphingomyelin, dipalmitoyl *NN*-dimethylphosphatidylethanolamine and phosphatidylserine. This type of interaction was not seen with chemically related but non-fusogenic lipids, nor was it found when the fusogenic lipids were mixed in monolayers with phosphatidylethanolamine. Our previous observations thus drew attention to the importance of a low-melting chain in the fusogenic lipid, and of the choline group in the phospholipid, for the occurrence of negative deviations of mean molecular area in the mixed monolayers studied.

In the present work the nature of interactions occurring in mixed monolayers of phospholipids and fusogenic lipids has been investigated further by studying the surface potentials of mixed monolayers. We have found that the presence in the subphase of bivalent cations, including Ca^{2+}, significantly modifies the properties of mixed monolayers containing fusogenic lipids and phosphatidylcholine or phosphatidylethanolamine. This and other findings indicate that the polar head group of the phospholipid is involved in the interactions of phospholipid molecules with fusogenic lipids. Since the bivalent cation ionophore, A23187, facilitates the fusion of hen erythrocytes (Ahkong *et al.*, 1975) and as Ca^{2+} is essential for the fusion of these cells by fusogenic lipids (Ahkong *et al.*, 1973), our observations indicate also that Ca^{2+} may modify the asymmetric physical properties of the phospholipid bilayer of hen erythrocytes when the cells fuse on treatment with fusogenic lipids.

Materials and Methods

The sources and purity of the lipids and phospholipids studied, the equipment used, and the preparation of the surface monolayers have been published previously (Maggio & Lucy, 1975). Dihexadecyl phosphate and octadecylamine were from K & K Laboratories, Plainview, NY, U.S.A. Hexadecyltrimethylammonium bromide of the purest grade, AnalaR NaCl, $CaCl_2$ and uranyl nitrate were from BDH, Poole, Dorset, U.K. NaCl was roasted at 400°C for 5h before use, and water was double-distilled (final distillation over alkaline $KMnO_4$) in

an all-glass apparatus. Measurements of surface potential were made (simultaneously with automatically recorded measurements of surface pressure and surface area), by using an air-ionizing electrode of ^{241}Am, which was suspended over the lipid monolayer (7mm from the air/water interface), and a calomel electrode which was connected through a salt bridge to the film-free subphase on the far side of the Teflon barrier. The trough was enclosed in an earthed metal box in which a N_2-enriched atmosphere was maintained. A Beckman Zeromatic pH-meter, functioning as a millivolt meter, was connected to both electrodes for the measurement of surface potential; the meter was adjusted to give a zero reading with a clean (lipid-free) air/subphase interface. The output from the meter was continuously recorded on a Beckman potentiometric recorder. Curves of surface potential versus area were run in duplicate or triplicate and were reproducible within 5–10mV; the replicate curves were averaged to yield values of surface potential. The force–area curves, which were obtained within 8min at a constant rate of compression (Maggio & Lucy, 1975), were run in duplicate and were reproducible within $\pm 0.02 nm^2$ per molecule and $\pm 1 mN \cdot m^{-1}$ ($\pm 1 dyn \cdot cm^{-1}$). Isotherms were measured after allowing 2min for evaporation of the solvent. All measurements were made at $23 \pm 1°C$ on subphases of unbuffered 145mM-NaCl (approx. pH5.6), containing other salts as indicated. When necessary, the pH of the subphase was adjusted to the desired value with HCl (5M) or NaOH (5M) before spreading the monolayer.

The surface behaviour of the different mixed monolayers was analysed by comparing the plots of surface pressure versus area, and surface potential versus area, with the theoretical curves for corresponding ideally mixed films. Areas per molecule in the ideal mixtures at each surface pressure studied were calculated, by using the additivity rule (Gaines, 1966), from the areas per molecule exhibited by the pure components at the same pressure. Surface potentials for the ideally mixed films were calculated from the ideal mean molecular areas and the additivity rule of surface potential per molecule (Shah, 1970). The formulae used were:

$$A_i = N_1 A_1 + N_2 A_2$$

$$\Delta V_i = \frac{[N_1(\Delta V/n)_1 + N_2(\Delta V/n)_2]}{A_i}$$

where A is the molecular area in cm^2, ΔV is the surface potential in mV, n is the number of molecules per cm^2 of surface and N the molar fraction of a component in the mixture; the subindices i, 1 and 2 refer respectively to the values for ideally mixed films and components 1 and 2 in individual monolayers at the same surface pressure.

Interactions of lipid monolayers with cations can make the films positively charged, especially with the uranyl ion, which shows a great affinity for the phosphate groups of phospholipids and probably binds quantitatively to them (Bangham *et al.*, 1967). Such changes might conceivably alter the contact angle and hence affect the validity of the surface-pressure measurements (Gaines, 1966). In some of the experiments with bivalent cations in the subphase, the surface pressure was therefore also determined by using a glass wire, as an alternative to the usual platinized Pt wire. The results obtained with the two types of sensing element were completely similar, indicating that the observed changes in surface pressure were not due to modifications of the contact angle.

Results

Surface potential, surface area and pH

The mean surface potential per molecule for mixed monolayers of glycerol mono-oleate and dipalmitoylglycerylphosphorylcholine, in different molar proportions on a subphase of unbuffered NaCl (145mM) at approx. pH5.6, showed negative deviations from the additivity rule (Fig. 1*b*). As reported previously (Maggio & Lucy, 1975), similar negative deviations were apparent for the mean area per molecule in these mixed monolayers (Fig. 1*a*). The results obtained with glycerol monostearate, which, unlike glycerol mono-oleate, is unable to cause cell fusion, followed ideality in mixed monolayers with dipalmitoylglycerylphosphorylcholine, both in mean area per molecule (Maggio & Lucy, 1975) and in surface potential.

In mixed monolayers of glycerol mono-oleate with dipalmitoylglycerylphosphorylethanolamine, the observed deviations from the additivity rule were positive, rather than negative, both for surface potential and, as found previously (Maggio & Lucy, 1975), for surface area (Fig. 2). Similar positive deviations were also obtained for mixed monolayers of the ethanolamine-containing phospholipid with glycerol monostearate.

Other fusogenic lipids, e.g. oleic acid (octadec-*cis*-9-enoic acid) and isostearic acid (methylheptadecanoic acid, mixture of isomers), behaved like glycerol mono-oleate in experiments with dipalmitoylglycerylphosphorylcholine and dipalmitoylglycerylphosphorylethanolamine. Stearic acid (octadecanoic acid), which is not fusogenic, behaved like glycerol monostearate. The observed negative and positive deviations from the additivity rule for surface potential indicate that ion–dipole interactions occur in the mixed monolayers, as well as changes in molecular packing that give rise to deviations from ideality in mean molecular area.

The participation of the phospholipid polar groups

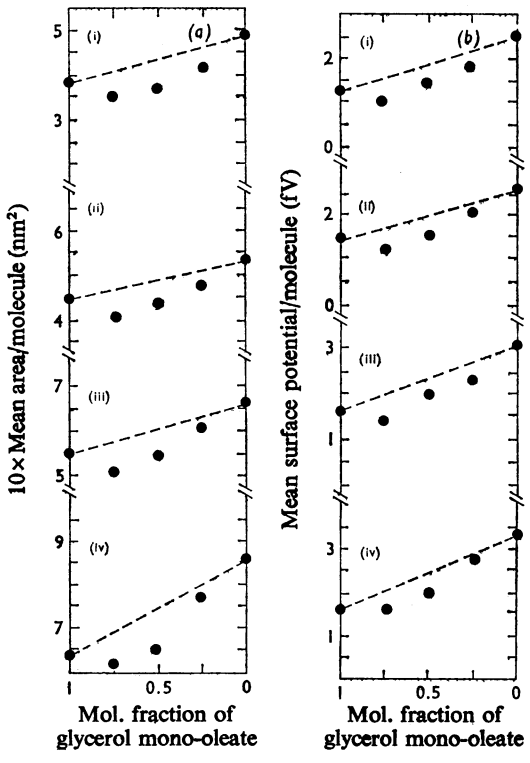


Fig. 1. *Mean area/molecule and mean surface potential/molecule in mixed monolayers of dipalmitoylglycerylphosphorylcholine and glycerol mono-oleate*

The mean area/molecule (*a*) and the mean surface potential/molecule (*b*) were determined, as described in the text, for mixed monolayers of dipalmitoylglycerylphosphorylcholine and glycerol mono-oleate on subphases of 145mM-NaCl, approx. pH5.6. Determinations were made at different surface pressures: (i) 30mN·m^{-1}; (ii) 20mN·m^{-1}; (iii) 10mN·m^{-1}; (iv) 5mN·m^{-1}. The broken lines represent values calculated by the additivity rule.

in the observed interactions was further investigated by varying the pH of the subphase. First, the effect of pH on the behaviour of one-component films was studied. Fig. 3 shows the surface pressure–area and surface potential–area curves obtained for dipalmitoylglycerylphosphorylcholine and for dipalmitoylglycerylphosphorylethanolamine at pH2, 5.6 and 10. These data are in general agreement with those in the literature, although strict comparisons are not possible as the experimental conditions were not identical (Shah & Shulman, 1967; Standish & Pethica, 1968; Vilallonga, 1968). Both phospholipids showed the characteristic increase in surface potential due to protonation of their primary phosphate group (Papahadjopoulos, 1968; Shah & Shulman, 1967). As expected, the isotherms obtained for one-component films of glycerol mono-oleate and of glycerol monostearate did not show any variation with pH. The curves for oleic acid, isostearic acid and stearic acid exhibited the behaviour to be expected on titration of their carboxylate groups (Goerke *et al.*, 1970).

The surface pressure–area and surface potential–area curves at pH2, 5.6 and 10 for mixed monolayers of the fusogenic lipid, glycerol mono-oleate, with dipalmitoylglycerylphosphorylcholine and with dipalmitoylglycerylphosphorylethanolamine are shown in Fig. 4. For simplicity, only the isotherms for equimolar mixtures are given: similar results were obtained with mixtures in the molar propor-

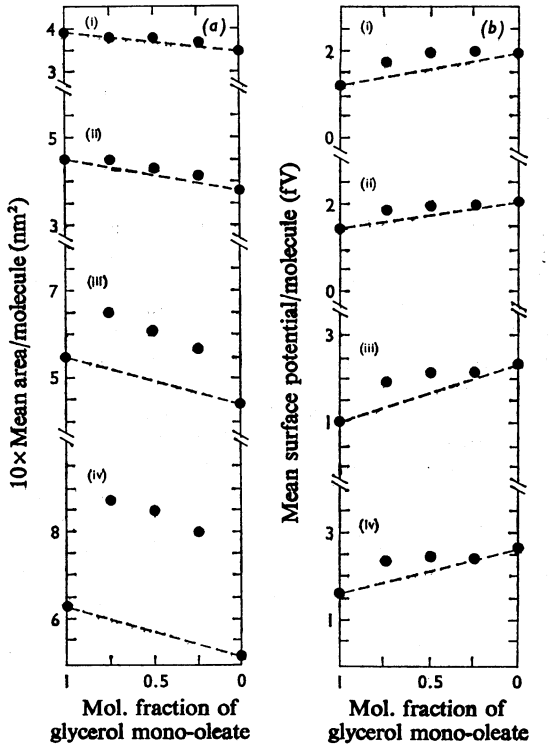


Fig. 2. *Mean area/molecule and mean surface potential/molecule in mixed monolayers of dipalmitoylglycerylphosphorylethanolamine and glycerol mono-oleate*

The mean area/molecule (*a*) and the mean surface potential/molecule (*b*) were determined, as described in the text, for mixed monolayers of dipalmitoylglycerylphosphorylethanolamine and glycerol mono-oleate on subphases of 145mM-NaCl, approx. pH5.6. Determinations were made at different surface pressures: (i) 30mN·m^{-1}; (ii) 20mN·m^{-1}; (iii) 10mN·m^{-1}; (iv) 5mN·m^{-1}. The broken lines represent values calculated by the additivity rule.

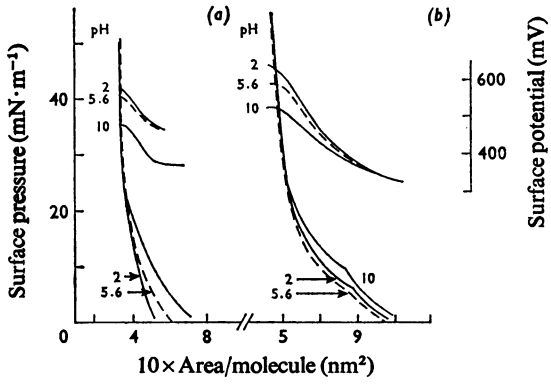


Fig. 3. *Influence of pH on the surface pressure–area and surface potential–area curves of dipalmitoylglycerylphosphorylethanolamine and dipalmitoylglycerylphosphorylcholine*

The isotherms for dipalmitoylglycerylphosphorylethanolamine (*a*) and dipalmitoylglycerylphosphorylcholine (*b*) were obtained on subphases of 145mM-NaCl adjusted to the indicated values of pH.

tions 3:1 and 1:3 (lipid/phospholipid). Fig. 4 shows that mixed monolayers of glycerol mono-oleate and dipalmitoylglycerylphosphorylcholine exhibited negative deviations with respect to both surface area and surface potential at pH5.6 and 10, but at pH2 nearly ideal behaviour was found. By comparison, mixed monolayers of glycerol monostearate and dipalmitoylglycerylphosphorylcholine gave ideal behaviour for both parameters at each pH (not shown). With dipalmitoylglycerylphosphorylethanolamine, glycerol mono-oleate and glycerol monostearate both gave positive deviations in surface area at pH5.6 and 10, but also exhibited almost ideal mixing at pH2; the surface potential-area curves of these mixed monolayers followed ideality at each pH studied (Fig. 4). Mixed monolayers of dipalmitoylglycerylphosphorylcholine and dipalmitoylglycerylphosphorylethanolamine with the fusogenic oleic acid and isostearic acid showed patterns of behaviour that were similar to those with glycerol mono-oleate, whereas stearic acid behaved like glycerol monostearate. The properties of mixed films of fusogenic and non-fusogenic lipids with natural phospholipids (egg phosphatidylcholine and egg phosphatidylethanolamine) were also studied at pH5.6 and 2. These mixed monolayers behaved like those containing the corresponding synthetic phospholipids (cf. Fig. 4).

The fact that negative deviations from ideality in surface potential and surface area, in mixed monolayers of both ionizable (e.g. oleic acid) and non-ionizable (e.g. glycerol mono-oleate) fusogenic lipids with dipalmitoylglycerylphosphorylcholine, were decreased on changing the pH of the subphase from pH5.6 to 2 indicates that the polar head group of the phospholipids is involved. It is also noteworthy that the differing behaviour shown in mixed films by choline-containing phospholipids (negative deviations in mean molecular area) and ethanolamine-containing phospholipids (positive deviations in mean molecular area) is eliminated at pH2, and that both species of phospholipid show almost ideal mixing behaviour at this pH with fusogenic and with non-fusogenic lipids.

Effects of bivalent cations on surface potential and surface area

The participation of the polar head groups of the phospholipid molecules was analysed further in in the following experiments by including bivalent cations in the subphase in addition to unbuffered 145mM-NaCl. In these studies, our aim was to see if interactions of bivalent cations with phospholipid head groups could modify the behaviour of mixtures of phospholipids with fusogenic or non-fusogenic lipids.

Increased surface potentials of monolayers of dipalmitoylglycerylphosphorylcholine and dipalmitoylglycerylphosphorylethanolamine were observed when these phospholipids were spread alone on subphases containing 10mM-Ca^{2+} or 1mM-UO_2^{2+} (Fig. 5); the values found were generally within the range reported by Shah (1969), Shah & Shulman (1965) and Standish & Pethica (1968). As with the effects of pH, a strict comparison with published data is not possible since the experimental conditions were not identical. The surface pressure–area isotherms of dipalmitoylglycerylphosphorylcholine and dipalmitoylglycerylphosphorylethanolamine in the presence of 10mM-Ca^{2+} were little different from those on a subphase free from Ca^{2+} (Fig. 5). In the presence of 1mM-UO_2^{2+}, however, the surface pressure–area isotherms of dipalmitoylglycerylphosphorylcholine and dipalmitoylglycerylphosphorylethanolamine were unusually alike, the areas per molecule having been decreased and increased respectively, at all values of surface pressure (Fig. 5), by the presence of the uranyl ion (cf. Hayashi *et al.*, 1972). Bivalent cations had no effect on the surface potential–area or surface pressure–area curves of either glycerol mono-oleate or glycerol monostearate.

In Fig. 6 are shown the data obtained for mixed monolayers spread on subphases containing 10mM-Ca^{2+} in addition to unbuffered 145mM-NaCl (adjusted to pH5.6). Ca^{2+} had little effect on mixed monolayers of glycerol mono-oleate (cf. Fig. 4; pH5.6) or glycerol monostearate with dipalmitoylglycerylphosphorylcholine. By contrast, mixtures of glycerol mono-oleate or glycerol monostearate with dipalmitoylglycerylphosphorylethanolamine showed

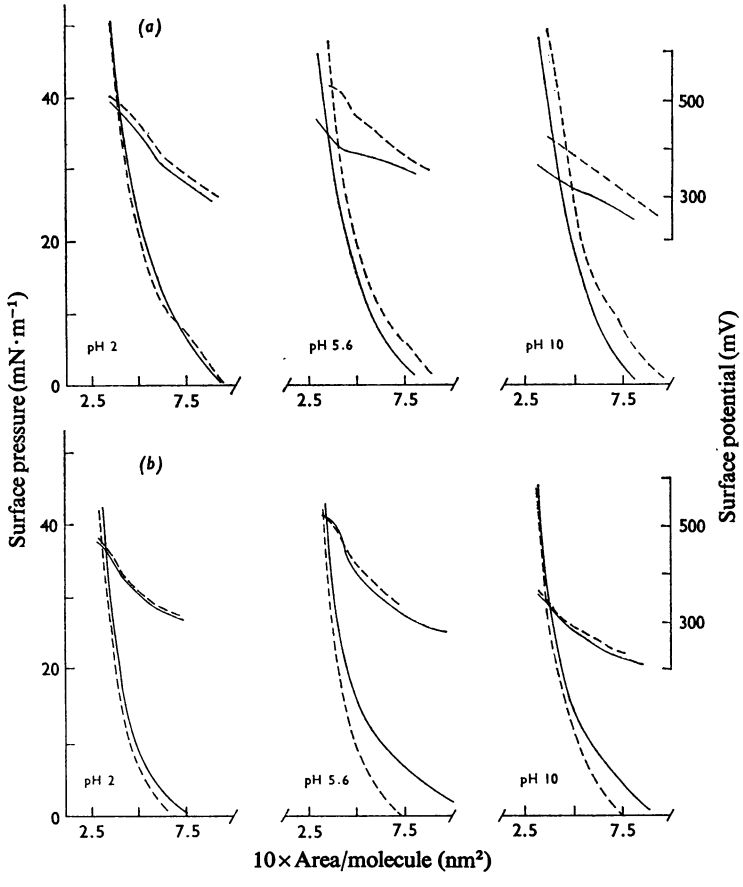


Fig. 4. *Influence of pH on the surface pressure–area and surface potential–area curves of mixed monolayers of glycerol mono-oleate with dipalmitoylglycerylphosphorylcholine or with dipalmitoylglycerylphosphorylethanolamine*

The isotherms for mixed monolayers of dipalmitoylglycerylphosphorylcholine (*a*) or dipalmitoylglycerylphosphorylethanolamine (*b*) with glycerol mono-oleate in a 1:1 molar ratio were obtained on subphases of 145mM-NaCl adjusted to the indicated values of pH. The dashed isotherms represent the values calculated by the additivity rule.

negative deviations in mean molecular area and surface potential in the presence of 10mM-Ca^{2+} at surface pressures above about 15mN·m^{-1}. In other words, when Ca^{2+} was present, mixed monolayers of dipalmitoylglycerylphosphorylethanolamine with either glycerol mono-oleate (fusogenic) or with glycerol monostearate (non-fusogenic) behaved at high surface pressures like mixed monolayers of the fusogen, glycerol mono-oleate, with a choline-containing phospholipid. Similar findings were made when the mixed monolayers were spread on a subphase of unbuffered 145mM-NaCl (pH5.6) which was subsequently adjusted to 10mM-Ca^{2+} by injecting the appropriate amount of 1M-$CaCl_2$, followed by stirring for 3min, before compressing the film. Experiments with Zn^{2+} gave results like those observed with Ca^{2+}.

Fig. 7 illustrates the corresponding results with 1mM-UO_2^{2+} in the subphase. Above surface pressures of about 20mN·m^{-1}, the behaviour of mixed monolayers of glycerol mono-oleate and glycerol monostearate with dipalmitoylglycerylphosphorylcholine was, as with Ca^{2+}, little affected by the bivalent cation. Also, similar to what was found in the presence of Ca^{2+} or Zn^{2+}, mixed monolayers of glycerol mono-oleate or glycerol monostearate with dipalmitoylglycerylphosphorylethanolamine showed (at high surface pressures) negative deviations in mean molecular area and surface potential like those observed in mixtures of the fusogen, glycerol mono-oleate, with the choline-containing phospholipid. However, negative deviations in mean molecular area (with glycerol mono-oleate, Fig. 4; pH5.6) and ideal mixing (with

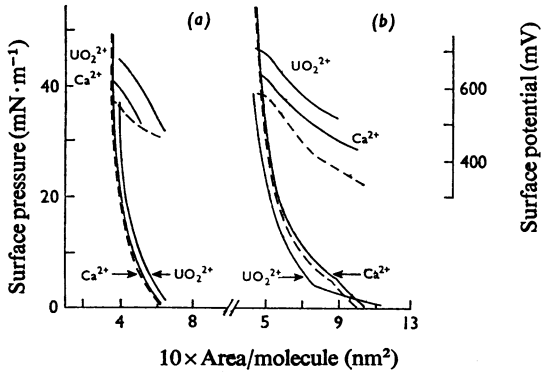


Fig. 5. *Surface pressure–area and surface potential–area curves of dipalmitoylglycerylphosphorylethanolamine and dipalmitoylglycerylphosphorylcholine on subphases containing bivalent cations*

The isotherms for dipalmitoylglycerylphosphorylethanolamine (*a*) and dipalmitoylglycerylphosphorylcholine (*b*) were obtained on subphases of 145mM-NaCl, adjusted to pH5.6, containing 10mM-$CaCl_2$ or 1mM-$UO_2(NO_3)_2$ as indicated. The dashed isotherms represent the values obtained on 145mM-NaCl, pH5.6, free from bivalent cations.

glycerol monostearate) were replaced by marked positive deviations in the presence of UO_2^{2+} with mixed monolayers containing dipalmitoylglycerylphosphorylcholine at pressures below about 20mN·m^{-1}. Similar results were obtained under these conditions with mixed monolayers containing dipalmitoylglycerylphosphorylethanolamine. With uranyl ions, unlike experiments with Ca^{2+}, injection of the bivalent cation into the subphase after the monolayer had already been spread on a NaCl subphase led to mixed monolayers containing dipalmitoylglycerylphosphorylethanolamine following, in general, the additivity rule for molecular area and surface potential. In these conditions monolayers containing dipalmitoylglycerylphosphorylcholine showed, above 20mN·m^{-1}, essentially the same behaviour as in Fig. 7. Below this surface pressure, positive deviations in molecular areas were still present but were decreased to about 30% of those shown in Fig. 7.

The behaviour of the mixed monolayers was dependent on the concentrations of cations used. Mixtures containing dipalmitoylglycerylphosphorylethanolamine did not exhibit the behaviour shown in Figs. 6 and 7 at concentrations of bivalent cation in the subphase below 1mM-Ca^{2+} or 0.1mM-UO_2^{2+}. Monolayers spread on subphases containing 0.1mM-Ca^{2+} behaved like those on NaCl alone; increasing the concentration of Ca^{2+} up to 100mM gave results like those obtained with 10mM-Ca^{2+}. Monolayers on 1–10μM-UO_2^{2+} were like those on NaCl alone; with 10mM-UO_2^{2+}, the results were as shown in Fig. 7.

Observations exactly similar to those of Figs. 6 and 7 were made in experiments with oleic acid and isostearic acid (fusogenic) and stearic acid (non-fusogenic), which, unlike glycerol mono-oleate and glycerol monostearate, possess polar groups that can interact with the bivalent cations (Shah, 1970). Hence, as in our previous studies (Maggio & Lucy, 1975) and in the findings reported above with monolayers at different pH, the polar groups of the lipid agents do not seem to be involved in the changes under study. By contrast, it appears that modifications in the properties of the polar head groups of the phospholipids, which occur when

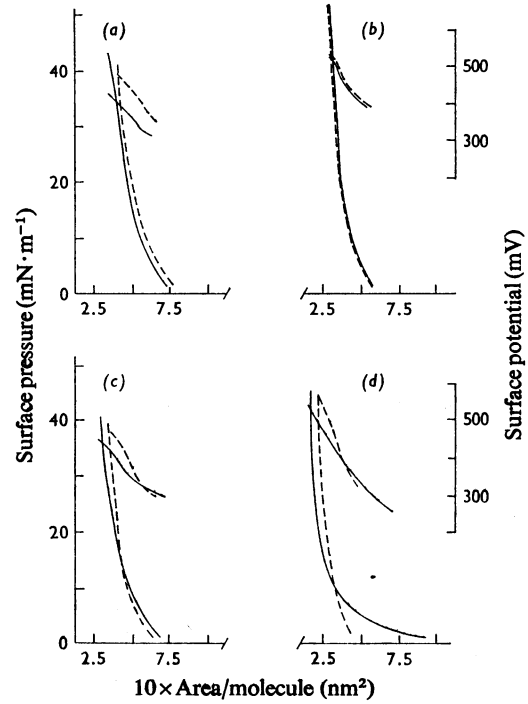


Fig. 6. *Effect of Ca^{2+} ions on the surface pressure–area and surface potential–area curves of mixed monolayers of glycerol esters with dipalmitoylglycerylphosphorylcholine or dipalmitoylglycerylphosphorylethanolamine*

The isotherms were obtained on 145mM-NaCl, adjusted to pH5.6 and containing 10mM-$CaCl_2$, for mixed monolayers of phospholipids and glycerol esters in 1:1 molar proportions. (*a*) and (*b*), Dipalmitoylglycerylphosphorylcholine with glycerol mono-oleate (*a*) or glycerol monostearate (*b*). (*c*) and (*d*), Dipalmitoylglycerylphosphorylethanolamine with glycerol mono-oleate (*c*) or glycerol monostearate (*d*). The dashed isotherms represent the values calculated by the additivity rule.

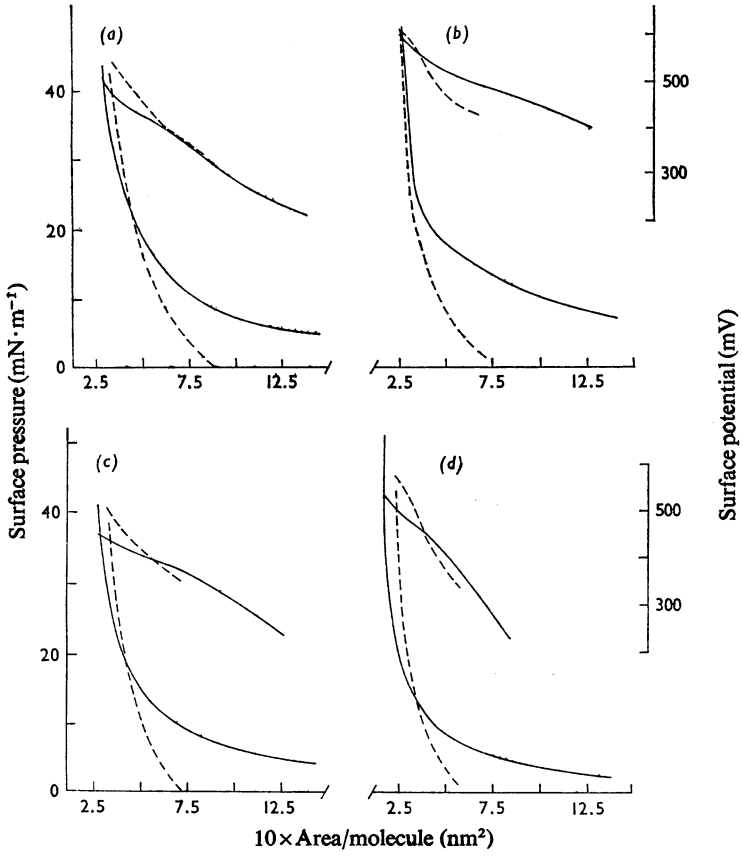


Fig. 7. *Effect of uranyl ions on the surface pressure–area and surface potential–area curves of mixed monolayers of glycerol esters with dipalmitoylglycerylphosphorylcholine or dipalmitoylglycerylphosphorylethanolamine*

The isotherms were obtained on subphases of 145mM-NaCl, adjusted to pH5.6 and containing 1mM-$UO_2(NO_3)_2$, for mixed monolayers of phospholipids and glycerol esters in 1:1 molar proportions. (*a*) and (*b*), Dipalmitoylglycerylphosphorylcholine with glycerol mono-oleate (*a*) or glycerol monostearate (*b*). (*c*) and (*d*), Dipalmitoylglycerylphosphorylethanolamine with glycerol mono-oleate (*c*) or glycerol monostearate (*d*). The dashed isotherms represent the values calculated by the additivity rule.

bivalent cations are present in the subphase, strongly influence the way in which phospholipid molecules interact in monolayers with fusogenic and non-fusogenic lipids.

Effects of lipids on the interactions of bivalent cations with phospholipids

The possibility that the presence of the fusogenic or non-fusogenic lipids in the mixed monolayer may, conversely, interfere with the interaction of bivalent cations with the head groups of phospholipid molecules was also investigated. This was studied by measuring the changes in surface potential that occurred with a series of 10-fold increases in the concentration of bivalent cation in the subphase (Davies & Rideal, 1963; Shah & Shulman, 1967); the results were compared with the changes in surface potential to be expected for an ideal film. The experiments were restricted to mixtures of non-ionizable glycerol mono-oleate or glycerol monostearate with dipalmitoylglycerylphosphorylcholine and dipalmitoylglycerylphosphorylethanolamine at pH5.6, to avoid introducing additional charges that would change the zwitterionic nature of the monolayer. Under these conditions, the net charge of the monolayer is zero, no net ionic double layer is formed (Davies & Rideal, 1963), and the slope of the line relating surface potential to $\log[M^{n+}]$ concentration is related to the affinity of the phospholipid head group for the cation (Shah & Shulman, 1967).

The data obtained at high surface pressure ($30mN \cdot m^{-1}$) are illustrated in Fig. 8 for subphases

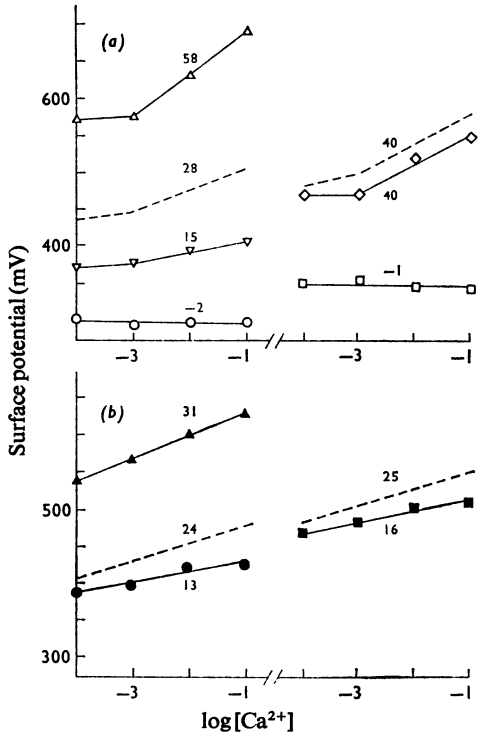


Fig. 8. *Effect of Ca^{2+} ions on the surface potential of mixed monolayers of phospholipids and glycerol esters*

The values of surface potential shown were derived (at a surface pressure of $30\,mN \cdot m^{-1}$) from appropriate surface potential–area isotherms on 145mM-NaCl, adjusted to pH 5.6 and containing the concentration of $CaCl_2$ indicated, for dipalmitoylglycerylphosphorylcholine (△), glycerol mono-oleate (○), glycerol monostearate (□), an equimolar mixture of dipalmitoylglycerylphosphorylcholine with glycerol mono-oleate (▽) or with glycerol monostearate (◇), dipalmitoylglycerylphosphorylethanolamine (▲), an equimolar mixture of dipalmitoylglycerylphosphorylethanolamine with glycerol mono-oleate (●) or with glycerol monostearate (■). The numbers indicate the slope ($mV \cdot l \cdot mol^{-1}$) of the curves (to the right of any inflexion point). The broken lines represent the values derived from the isotherms calculated by the additivity rule.

containing Ca^{2+}. For mixtures of glycerol mono-oleate with dipalmitoylglycerylphosphorylcholine, and for either glycerol monostearate or glycerol mono-oleate with dipalmitoylglycerylphosphorylethanolamine, the slopes of the experimental curves were less than those calculated for ideal films, indicating a decrease in the interaction of Ca^{2+} with the phospholipid. By contrast, the behaviour of glycerol monostearate with dipalmitoylglycerylphosphorylcholine was practically ideal. A similar series of observations were made in experiments with UO_2^{2+}. It is thus apparent that, except for glycerol monostearate with dipalmitoylglycerylphosphorylcholine, lipid molecules in the mixed monolayers are able to modify the interaction of bivalent cations with the phospholipids, presumably by altering the orientation of phospholipid dipoles.

These findings are commensurate with the data on mean molecular areas in mixed monolayers in the presence of Ca^{2+} (Fig. 6), from which it is apparent that the only mixed monolayer to exhibit ideal behaviour at $30\,mN \cdot m^{-1}$ is that of glycerol monostearate with dipalmitoylglycerylphosphorylcholine.

Dihexadecyl phosphate and hexadecyltrimethylammonium bromide

Experiments were undertaken to find whether

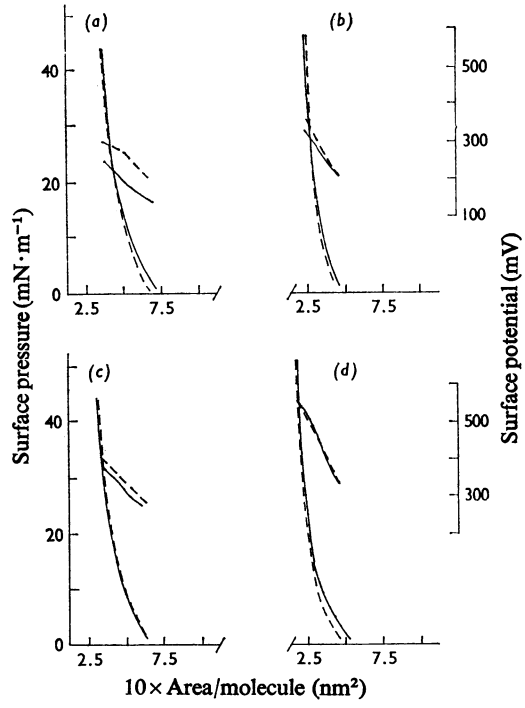


Fig. 9. *Surface pressure–area and surface potential–area curves of mixed monolayers of glycerol esters and dihexadecylphosphate and hexadecyltrimethylammonium*

Isotherms were obtained on subphases of 145mM-NaCl, adjusted to pH 5.6, for mixed monolayers containing 1:1 molar ratios of dihexadecyl phosphate with glycerol mono-oleate (*a*) or with glycerol monostearate (*b*). Curves are also shown for a 1:1 molar mixture of dihexadecyl phosphate and hexadecyltrimethylammonium in equimolar proportions with glycerol mono-oleate (*c*) or with glycerol monostearate (*d*). The dashed isotherms represent the values calculated by the additivity rule.

interactions similar to those reported above could also occur in mixed films containing molecules that each possess one, but not both, of the charged groups that are present in phospholipid molecules.

Closely packed films of dihexadecyl phosphate had a limiting area of about 0.38 nm^2 per molecule (similar to dipalmitoylglycerylphosphorylcholine; 0.44 nm^2) and a surface potential of 290 mV, in accordance with the values reported by Parreira & Pethica (1957) and Shah & Shulman (1965). Hexadecyltrimethylammonium exhibited an expanded type of isotherm (Davies & Rideal, 1963). An equimolar mixture of these two substances at surface pressures below 30 $mN \cdot m^{-1}$ on a subphase of unbuffered 145 mM-NaCl (adjusted to pH 5.6) showed considerable decreases in mean molecular area with respect to an ideal film, resulting from mutual charge neutralization and a consequent decrease in intermolecular electrostatic repulsion. At higher pressures, the additivity rule was followed because the molecular areas of the component molecules in the mixed film could not be decreased further; the ionic interactions between the polar head groups were then maximal, giving a surface potential that was about 200 mV greater than the value for ideal mixing.

A similar behaviour was noted with a mixed monolayer of dihexadecyl phosphate and octadecylamine.

In mixed monolayers of glycerol mono-oleate with dihexadecyl phosphate, ion–dipole interactions occurred, as shown by a negative deviation in surface potential per molecule, but there was no decrease in the mean molecular area (Fig. 9*a*). The negative deviation in surface potential was itself almost abolished when glycerol mono-oleate was replaced by glycerol monostearate, despite the polar moiety being unchanged. When glycerol mono-oleate was mixed in equimolar proportions with a 1:1 molar mixture of dihexadecyl phosphate/hexadecyltrimethylammonium (or dihexadecyl phosphate/octadecylamine) a small ion–dipole interaction was seen, but no deviations in mean molecular area occurred (Fig. 9*c*). This mixed monolayer contains components essentially similar to those in a mixed film of glycerol mono-oleate and dipalmitoylglycerylphosphorylcholine. A comparison with Fig. 4 (pH 5.6) shows, however, that the molecular interactions occurring in the two systems are quite different.

The behaviour of these mixtures was also studied on subphases containing 10 mM-Ca^{2+}. It was observed, in confirmation of the findings of Shah & Shulman

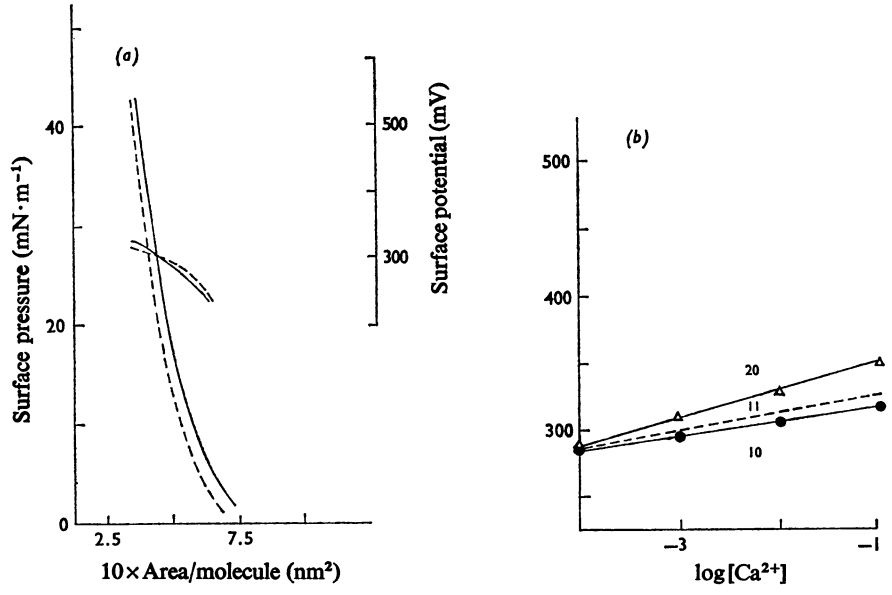


Fig. 10. *Effect of Ca^{2+} ions on interactions between dihexadecyl phosphate and glycerol mono-oleate*

The isotherms were obtained on subphases of 145 mM-NaCl, adjusted to pH 5.6. Surface pressure–area and surface potential–area curves of an equimolar mixture of dihexadecyl phosphate and glycerol mono-oleate on a subphase containing 10 mM-$CaCl_2$ are shown in (*a*). Surface potential–concentration curves are shown in (*b*): the values of surface potential shown were derived (at a surface pressure of 30 $mN \cdot m^{-1}$) from appropriate surface potential–area isotherms on subphases containing the concentration of $CaCl_2$ indicated, for dihexadecyl phosphate (△) or an equimolar mixture of glycerol mono-oleate and dihexadecyl phosphate (●). The numbers indicate the slope of the curves. The dashed lines represent the values calculated by the additivity rule.

(1967), that the interaction of the cationic group of hexadecyltrimethylammonium with the polar group of dihexadecyl phosphate prevents Ca^{2+} from binding to dihexadecyl phosphate in a mixed monolayer of these two substances. Conversely (Fig. 10*a*), the presence of Ca^{2+} ions in the subphase below a mixed monolayer of dihexadecyl phosphate with glycerol mono-oleate abolished the ion–dipole interactions between the two components of the monolayer which were seen in Fig. 9(*a*). Thus, as might be expected, the slope of the experimental binding curve for Ca^{2+} to a mixed monolayer of glycerol mono-oleate and dihexadecyl phosphate was almost ideal (Fig. 10*b*), showing that glycerol mono-oleate had little effect on the binding of Ca^{2+} by dihexadecyl phosphate.

Discussion

The observations made in the present paper on mixed monolayers of lipids and phospholipids with respect to surface potential complement those reported previously for mean molecular areas (Maggio & Lucy, 1975). With both parameters, low-melting lipids that are fusogenic for hen erythrocytes (Ahkong *et al.*, 1973) are seen to interact differently from non-fusogenic lipids in mixed monolayers with phosphatidylcholine. Fusogenic and non-fusogenic lipids behave similarly, however, in mixed monolayers with phosphatidylethanolamine.

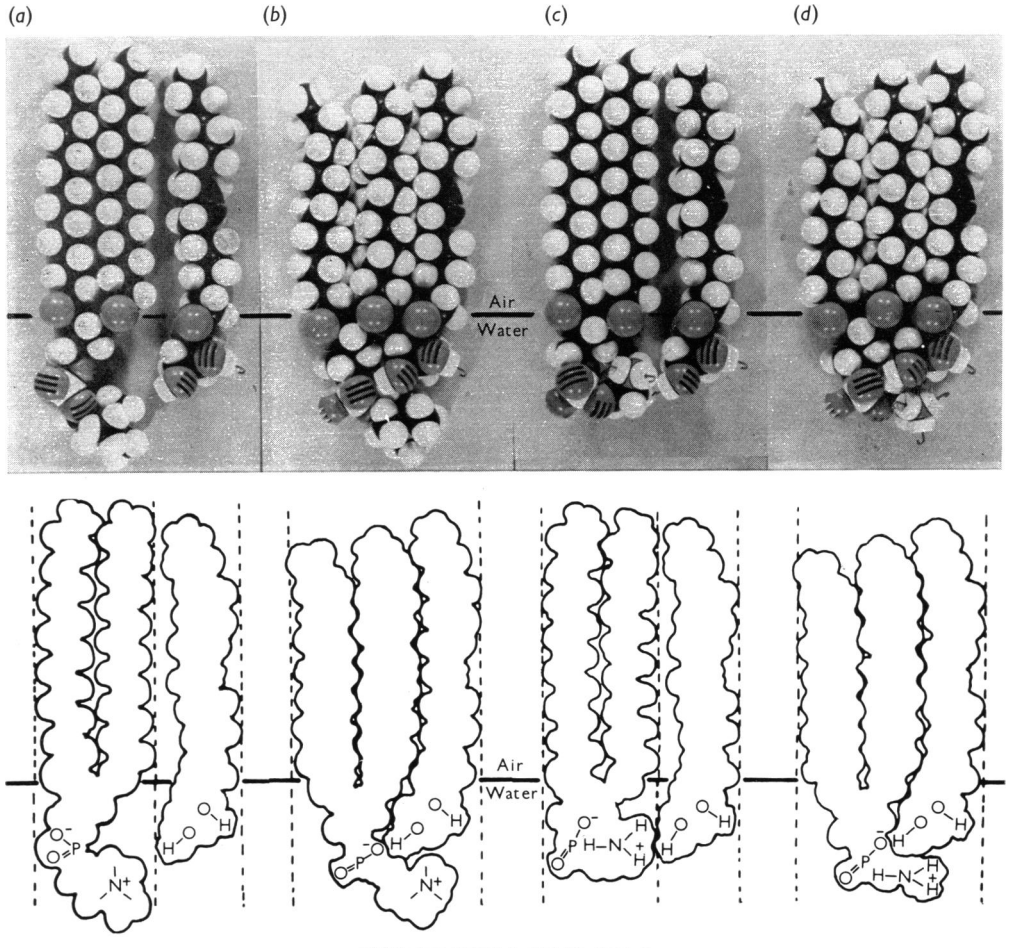
What is the molecular basis for the specific behaviour of fusogenic lipids with phosphatidylcholine, and why is this not seen with phosphatidylethanolamine? From the outset, it appears that the polar group of the lipid is relatively unimportant, since both glycerol mono-oleate and oleic acid are fusogenic, whereas both glycerol monostearate and stearic acid are not. This suggests that the specific surface properties of mixed monolayers of low-melting fusogenic lipids with phosphatidylcholine are to be attributed to an effect of the hydrocarbon chains of the fusogenic lipids which is mediated by the behaviour of the polar head group of phosphatidylcholine. For some reason, the polar head group of phosphatidylethanolamine behaves differently.

These conclusions, which are based only on considerations of molecular specificity, are supported by our investigations of the effects on monolayer behaviour of changes in pH. In mixtures of glycerol mono-oleate with egg phosphatidylcholine or dipalmitoylglycerylphosphorylcholine, the virtual abolition of negative deviations in surface potential and surface pressure that occurred on lowering the pH from 5.6 to 2 (Fig. 4) may be attributed to titration of the phosphate group of the phosphatidylcholine. At pH2 discrimination between fusogenic and non-fusogenic lipids in their behaviour in monolayers with phosphatidylcholine is lost, as is the differentiation between phosphatidylcholine and phosphatidylethanolamine, since all mixtures showed ideal behaviour for both surface pressure and surface potential at this pH (Fig. 4).

Interestingly, the 1:1 mixture of dihexadecyl phosphate and hexadecyltrimethylammonium showed very small negative deviations in surface potential with glycerol mono-oleate (and not with glycerol monostearate), and it did not exhibit negative deviations in mean molecular area with the fusogenic lipid (compare Fig. 9*c* with Fig. 4). Presumably, intermolecular interactions between the negative and positive charges of dihexadecyl phosphate and hexadecyltrimethylammonium are less readily perturbed by lipid esters in the monolayer than are interactions between the corresponding groups in a complete phospholipid molecule. These studies indicated that the complete polar group of phosphorylcholine (with two CH_2 groups between the positive and negative charges) is involved in its interactions with the fusogenic lipids, and not simply the individual charged components.

Our experiments with subphases containing Ca^{2+} or UO_2^{2+} have shown that cation binding affects the surface behaviour of the mixed monolayers in a complex way, depending not only on the species of cation and its concentration but even on the manner of its addition. From these studies, it is apparent that whether or not phosphatidylcholine interacts differentially with fusogenic and non-fusogenic lipids, and whether phosphatidylethanolamine behaves in the same way or differently, depends on the surface pressure of the system and on the degree to which the ion–dipole behaviour of the phospholipid is constrained by the influence of an external bivalent metal ion.

What is the converse effect of lipids on the ion–dipole behaviour of the polar groups of phospholipids in mixed monolayers? A comparison of Figs. 6 and 8 shows that the lipids altered the interactions of Ca^{2+} with phospholipid molecules. Only the phospholipid of the mixed monolayer that exhibited ideal behaviour (glycerol monostearate with dipalmitoylglycerylphosphorylcholine; Fig. 6*b*) had an ideal slope for the plot of surface potential against $\log[Ca^{2+}]$ (Fig. 8). Although changes in surface potential are difficult to interpret in absolute molecular terms, since ΔV is a quantity resulting from several non-determinable independent contributions (Davies & Rideal, 1963), some conclusions are possible, on a comparative basis, with mixed films containing molecules having the same polar moieties. We suggest that modification by fusogenic lipids of interactions between bivalent cations and phospholipids may not be a direct effect, but might be mediated by favouring closer intramolecular or intermolecular interactions of the



EXPLANATION OF PLATE I

Molecular models of glycerol mono-oleate and phospholipids

Space-filling Corey–Pauling–Koltun molecular models illustrating some possible molecular interactions between glycero mono-oleate and phospholipids. Outline projections of the models are also shown to facilitate the illustration of molecular and polar moiety volumes (vertical dashed lines). The molecules are considered to be in a closely packed state (i.e. above a surface pressure of $30 mN \cdot m^{-1}$). Equimolar mixture of glycerol mono-oleate and dipalmitoylglyceryl-phosphorylcholine: exhibiting theoretical ideal behaviour (cf. the Materials and Methods section) according to the additivity rule (*a*), illustrating a possible molecular arrangement giving non-ideal mixing (*b*). Equimolar mixture of glycerol mono-oleate and dipalmitoylglycerylphosphorylethanolamine: exhibiting ideal behaviour according to the additivity rule (*c*), illustrating a possible molecular arrangement giving non-ideal mixing (*d*). In (*a*) and (*c*) the phospholipids are represented with the polar head groups orientated according to Phillips *et al.* (1972) and with their acyl chains in an all-*trans* configuration. In (*b*) and (*d*) rotational isomers (three '2 gl kinks'; cf. Traüble & Haynes, 1971; Seelig & Seelig, 1974) are illustrated in each of the phospholipid acyl chains that allow co-operative close-packing with the fusogenic lipid (shown with three '2 gl kinks') consequent on ion–dipole interactions between a hydroxyl group of glycerol mono-oleate and the anionic oxygen in the polar head group of the phospholipid. Interactions of this type do not occur with phosphatidylethanolamine (*c*), until after the normal orientation of the polar moiety of this phospholipid has been disturbed (*d*) by bivalent cations in the subphase (see the text). The position of the air/water interface shown is intended to be illustrative and not definitive

negatively and positively charged groups of individual or adjacent phospholipid molecules. This idea is supported by the fact that a similar modification of bivalent-cation binding in mixed monolayers was seen, but to a much greater degree, in the total failure of dihexadecylphosphate to interact with Ca^{2+} when hexadecyltrimethylammonium was present in the monolayer. (Glycerol mono-oleate, by contrast, is unable to modify the interactions of dihexadecyl phosphate with Ca^{2+}; Fig. 10.) It is relevant to our hypothesis to note that unsaturated molecules of phosphatidylcholine show a lower affinity for Ca^{2+} than do saturated molecules; this has been attributed to a strengthened intramolecular salt linkage between phosphate and the trimethylammonium group in the unsaturated phosphatidylcholine (Shah & Shulman, 1965, 1967).

The presence of a low-melting fusogenic lipid in a mixed monolayer with phosphatidylcholine may facilitate the possibilities for molecular movement in the polar region of the phospholipid (Traüble & Haynes, 1971; Seelig & Seelig, 1974), such that a new and more stable arrangement of dipoles is produced (showing negative deviations in surface potential) which, in turn, allows closer packing of the components in the monolayer. In this new arrangement, the interactions of the phospholipid head group with bivalent cations are decreased. A relatively rigid saturated lipid, such as glycerol monostearate (non-fusogenic), would not produce this effect in a mixed monolayer with phosphatidylcholine. Also, for a fusogenic lipid to modify the properties of phosphatidylethanolamine molecules, in which intermolecular ionic interactions are probably greater than with phosphatidylcholine (Phillips *et al.*, 1972), it appears to be necessary to disturb the orientation of the polar moieties of the phospholipid monolayer by adding bivalent cations to the subphase. Once this has been done, both fusogenic and non-fusogenic lipids are then equally effective in modifying molecular packing. Plate 1 shows some of these possibilities with space-filling Corey–Pauling–Koltun molecular models: alternative structural arrangements of the interacting molecules are possible, however, and those shown should be regarded as illustrative and not definitive. In addition, other changes in molecular characteristics (e.g. in hydration or in the magnitudes of the fundamental dipoles) might be involved.

Fusogenic lipids are able to induce morphological changes (not given by non-fusogens) with liposomes of phosphatidylcholine, sphingomyelin or erythrocyte lipids (Howell *et al.*, 1973) which are probably related to the observed interactions in mixed monolayers. Also, Kantor & Prestegard (1975) have reported that myristic acid and lauric acid, but not palmitic acid, can induce fusion of vesicles of phosphatidylcholine, in agreement with the actions of these lipids on hen erythrocytes (Ahkong *et al.*, 1973). If the actions of fusogenic lipids in such model systems are, in fact, relevant to cell membranes, the findings reported in the present paper have some implications for membrane fusion. When a fusogenic lipid is introduced into the asymmetric bilayer structure of an erythrocyte membrane (Zwaal *et al.*, 1973), it may initially interact with choline-containing phospholipids in the outer half of the bilayer (Maggio & Lucy, 1975). As well as altering the polar head groups, the presence of low-melting molecules of fusogenic lipid might also co-operatively increase the number of rotational isomers (Traüble & Haynes, 1971) in clusters of hydrocarbon chains in the membrane interior. This would give a well-ordered bilayer containing disordered hydrocarbon chains (Seelig & Seelig, 1974). These changes may produce an increase in the permeability of the lipid bilayer (Traüble & Haynes, 1971; Marsh, 1974), thereby raising the concentration of Ca^{2+} within the cell (cf. Ahkong *et al.*, 1975). On the basis of our findings, it is interesting to speculate that in the presence of a fusogenic lipid the properties of the inner half of the lipid bilayer of the erythrocyte membrane (relatively rich in phosphatidylethanolamine) could well be altered, and behave like the outer half of the bilayer (relatively rich in phosphatidylcholine) (cf. Fig. 6), if the concentration of cytoplasmic Ca^{2+} is raised to about 1 mM. This increase in symmetry in the membrane might lower energy barriers for the intermixing of the constituents of membranes in closely adjacent erythrocytes and facilitate cell fusion.

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