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# SURFACE POTENTIALS AND ION SORPTION OF DIMYRISTOYL-, DIPALMITOYL- AND DISTEAROYL PHOSPHATIDYLCHOLINE FILMS ON NaCl AND $CaCl_2$ SOLUTIONS

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## Summary

When either dimyristoyl-, dipalmitoyl-, or distearoyl phosphatidylcholine was spread on the aqueous phase, the electrical potential at collapse pressure on 75 mM $CaCl_2$ was about 70 mV higher than on 150 mM NaCl; the difference between $Ca^{2+}$ and $Na^+$ fell gradually to 15—25 mV at the lower pressures. When, however, the electrolyte was injected under the dipalmitoyl phosphatidylcholine film preformed on distilled water, the surface potential at any film pressure was the same on water, NaCl or $CaCl_2$ in the first 4 min; but, the presence of $\geqslant 1$ mol% acidic phospholipid (dicetyl phosphate or dipalmitoyl phosphatidic acid), together with dipalmitoyl phosphatidylcholine in mixed films, caused the surface potential to increase rapidly in the order $CaCl_2 >$ NaCl $>$ water, by a quantity that was proportional to the concentration of the acidic lipid in the film and $Na^+$ or $Ca^{2+}$ in the aqueous phase. It is suggested that, in the absence of acidic phospholipids (contaminants), the modest rise in surface potential is due to penetration of the electrolyte's, as well as water's, ion pairs.

Measurements of surface radioactivity ($^{45}Ca^{2+}$) and infrared spectra of the interfacial material led to the discovery of a new species, $Ca(OH)_2$, at the air-water interface of $CaCl_2$ solutions. A direct correlation exists between adsorption of $Ca^{2+}$ and $Ca(OH)_2$ formation, both of which are enhanced by the presence of dipalmitoyl phosphatidylcholine at the interface; no specific correlation, however, can be established between either surface potential and ion binding of the neutral phospholipid or surface potential and ion pair penetration, since it is not yet possible to identify the electrically significant ion pair in the ionic maze of the interfacial lattice.

## Introduction

It has been generally observed that when phosphatidylcholine is spread on aqueous media, the surface potential ($\Delta V$) varies with both the nature ($H_2O <$

NaCl < $CaCl_2$) and the concentration of the electrolyte [1—4]. The greater surface potential on $CaCl_2$, as compared to NaCl, was attributed to a greater interaction of the electrolyte's cation ($Ca^{2+} > Na^{+}$) with either the polarized phosphate group of the choline phosphate zwitterion [1] or some ionic impurities [3,4]. Hauser and Phillips showed that, with films of phosphatidylcholine, both surface potential and adsorption of radioactive $Ca^{2+}$ increased with the $Ca^{2+}$ concentration in the hypophase; in contrast, although no $Ca^{2+}$ was adsorbed onto films of triolein, which is not ionic, the $\Delta V$ values increased with the $Ca^{2+}$ concentration [5]; they therefore suggested that the surface potential effect of $Ca^{2+}$ cannot be explained simply by the binding of $Ca^{2+}$ to the lipid and it is rather the result of various contributions which cannot as yet be evaluated individually. The conclusions reached more recently by a variety of techniques are discordant [6,7]. A new approach must therefore be sought, in order to explain the adsorption and surface potential data of $Ca^{2+}$ with monolayers of phosphatidylcholine.

We conducted two types of experiments. In one, as it is customary, we spread the phosphatidylcholine film on the electrolyte solution. In the other, we spread first the lipid film on distilled water, and then injected the electrolyte in the aqueous phase. Also, in order to identify the species adsorbed at the interface, we measured surface radioactivity using $^{45}Ca$ and $^{36}Cl$ radionuclides in the aqueous phase, and obtained the infrared adsorption spectra of dipalmitoyl phosphatidylcholine films spread onto NaCl and $CaCl_2$ solutions.

## Experimental procedures

### *Materials*

Cholesterol, and palmitic and stearic acids were purchased from Supelco, Belefonte, Pa. Synthetic dipalmitoyl phosphatidic acid, L-α-dimyristoyl-, and distearoyl phosphatidylcholines were purchased from Applied Science, State College, Pa.,; dipalmitoyl phosphatidylcholine and the sodium salt of dicetyl (hexadecoyl or palmitoyl) phosphate were Sigma products (Sigma Chemical Co., St. Louis, Mo.). After resolution of 250 μg lipid on precoated thin layer plates (Silica gel G, Analtech, Newark, Del.) in chloroform/methanol/conc. $NH_4OH$ (65 : 35 : 10, by vol.), and upon staining with $I_2$ vapors for lipid, with molybdenum oxide for the phospholipid, and with ninhydrin for free amino groups [3,4], each lipid preparation migrated as a single spot of probably homogeneous material. Thin layer chromatography, phosphorus and other qualitative and quantitative group analyses, used to monitor homogeneity and concentrations of the lipids, have been described [3,4,8—10]. In selected experiments, the lipid was fractionated on a column, using Unisil silicic acid (200—325 mesh) and various chloroform/methanol mixtures [3,4]; the thin layer and phosphorus analysis patterns of the rechromatographed lipids were identical to those before column chromatography.

The aqueous radiotracers $^{45}CaCl_2$ (410 μg/ml, specific activity 24.4 mCi/mg) and $H^{36}Cl$ (3.5 M, specific activity 0.51 mCi/ml) were obtained from New England Nuclear, Boston, Mass. To prepare $Ca^{36}Cl_2$ and $Na^{36}Cl$ stock solutions, the $H^{36}Cl$ was titrated with the required quantities of NaOH solution, and the neutral solution was then mixed with the desired volumes of nonradioactive $CaCl_2$ and NaCl solutions.

The organic solvents were spectral grade and were not redistilled before use. Water was distilled twice, the second time from alkaline permanganate. To avoid permanganate spraying or creeping into the condenser, we used a distillation flask with a long neck (30 cm), upon which was mounted upright a fractionating Vigreux column (40 cm). The conductivity of the resulting water was 1.0 $\mu\Omega^{-1}$/cm at 25°C [11]. The salts were reagent grade. The salt solutions were foamed in order to remove the surface active contaminants that are ordinarily present in reagent grade salts [11]. The lipid solutions, 1 mg/ml in 85 : 15 chloroform/methanol, were stored at —20°C between experiments, and were held in ice during the monolayer experiments; they were discarded after two weeks.

### *Monolayer techniques*

The trough consisted of a circular crystallizing dish, Pyrex glass, with an area of 26 cm$^2$. The experiments were carried out at constant area by measurement of the surface parameters as a function of the quantity of lipid applied onto the surface [8].

Procedures for the determinations of surface pressure $\pi$ (as the difference between two surface tension measurements, $\pi = \gamma_0 - \gamma$) and surface potential, $\Delta V = V - V_0$, have been described [8,11,12]. Surface radioactivity was measured by standard methods, using a Geiger counter (Eberline Instrument Corp., Santa Fe, New Mexico; Mod HP-190) connected to a scaler-ratemeter, Packard, Model 150. The Geiger tube had a thin mica window of 7 cm$^2$ useful area; a heating coil was wrapped around the tube in order to avoid absorption of radiations by condensing water vapor [13]. Calibration with increasing concentrations of either $^{45}CaCl_2$, $Ca^{36}Cl_2$ or $Na^{36}Cl$ in the aqueous phase produced the usual linear relationship of cpm to radionuclide concentration [5].

Infrared absorption spectra were obtained by attenuated total reflectance (ATR) on a germanium 30° angle plate, (Perkin Elmer, Norwalk, Conn.), using either films deposited directly on the plate or films lifted from the air/water interface [12].

## Results and Discussion

The surface pressure ($\pi$), surface potential ($\Delta V$) and surface radioactivity values are averages from at least three experiments. The average deviations were $<\pm0.5$ dyne/cm for the pressure and $<\pm5$ mV for the potential; the deviations for the radioactivity, in cpm, were larger for $^{45}Ca$ than for $^{36}Cl$.

### *Lipid films spread on electrolyte solution*

In line with previous data [1,2,14], at 25°C, the molecular areas varied in the order dimyristoyl- > dipalmitoyl- >> distearoyl phosphatidylcholine. With the three lipids, the surface potential on $CaCl_2$ was higher than on NaCl at all values of the molecular area (Fig. 1), and increased with the $Ca^{2+}$ concentration (Fig. 2). The interpretation of differences among the three phosphatidylcholines must be postponed until the molecular meaning of surface tension and surface potentials is understood.

Addition of up to 5 mol% of either palmitic acid or stearic acid and less than

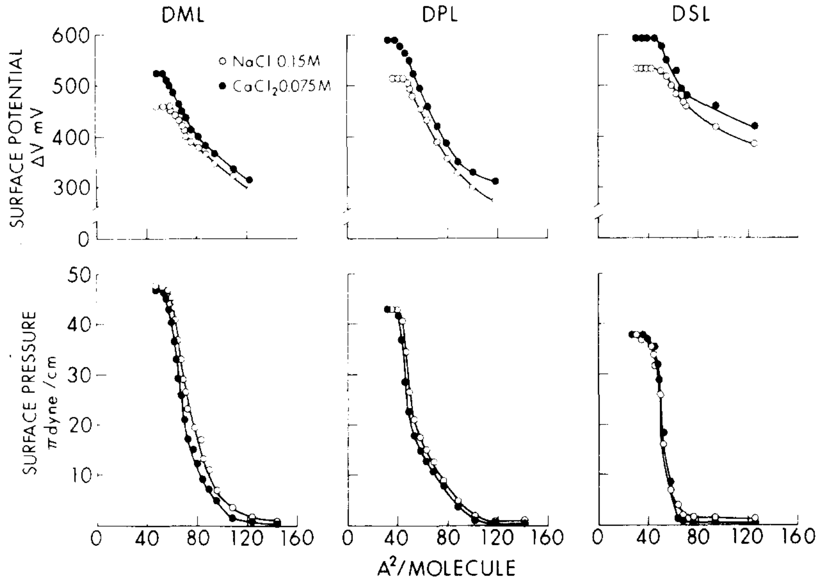

Fig. 1. Surface pressure-area and surface potential-area curves of dimyristoyl-, dipalmitoyl-, and distearoyl phosphatidylcholine on 150 mM NaCl (○) and 75 mM $CaCl_2$ (●), 25°C.

1 mol% dicetyl phosphate or dipalmitoyl phosphatidic acid did not cause appreciable deviations from the force area and $\Delta V$-area curves presented in Fig. 1. Also, the surface potential values for the phosphatidylcholine did not change significantly, whether the phospholipids were used directly or after rechromatography on silicic acid column. These results differ from previous ones [3,4], according to which rechromatography of the phospholipid on silicic acid columns caused the difference in surface potential between $CaCl_2$, NaCl

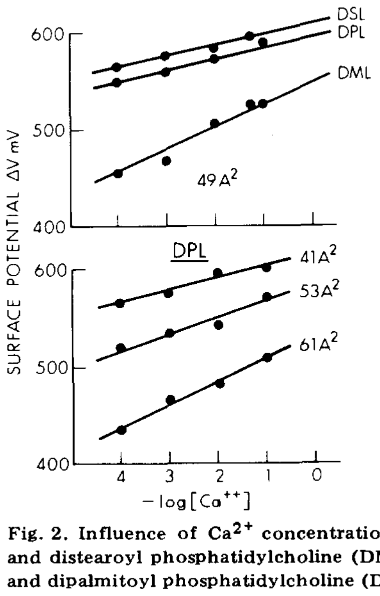

Fig. 2. Influence of $Ca^{2+}$ concentration at 25°C on the surface potentials of dimyristoyl-, dipalmitoyl- and distearoyl phosphatidylcholine (DML, DPL and DSL, respectively) at 49 $A^2$/molecule, (upper panel) and dipalmitoyl phosphatidylcholine (DPL) at 3 different molecular areas (lower panel).

and water to vanish, albeit the effect of the cationic contaminants of sphingomyelin [4] were reproduced. We have no explanation for the discrepancies in the case of phosphatidylcholine, except that now we have totally different lipid and also different Unisil preparations as compared to the earlier experiments [3,4]. Work in progress is aimed at showing how discrepancies can be produced by variations in experimental procedures.

### *Electrolyte injection under dipalmitoyl phosphatidylcholine films preformed on distilled water*

Unlike with phosphatidylcholine films spread onto the electrolyte solution, no difference in surface potential between water, NaCl and $CaCl_2$ was observed when the electrolyte at any concentration was injected under films of dipalmitoyl phosphatidylcholine that had been spread on distilled water (Fig. 3, left). However, when an acidic phospholipid, either dicetylphosphate (Fig. 3, right) or dipalmitoyl phosphatidic acid was mixed with the phosphatidylcholine, injection of the electrolyte in the distilled water hypophase, under the preformed mixed film, caused a marked and prompt rise in surface potential over that on water. This $\Delta V$ effect increased with the concentrations of both the acidic phospholipid (between 1 mol% and 10 mol%) and the electrolyte, and with the valency of the cation ($Ca^{2+} > Na^+$). One could speculate that, in the absence of acidic phospholipid, the $\Delta V$ increase could be due to penetration of ± oriented ion pairs of the electrolyte, water, or other species which are entrapped in the phosphatidylcholine film during spreading

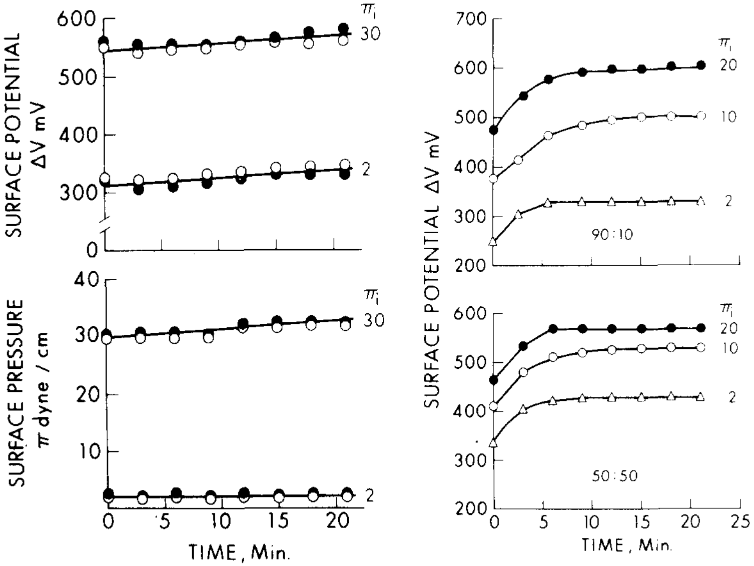

Fig. 3. Left panel: kinetic curves of the effect of $Na^+$ (○) and $Ca^{2+}$ (●) on the surface pressure and surface potential after injection of NaCl to 150 mM or $CaCl_2$ to 75 mM (final concentration) under films of dipalmitoyl phosphatidylcholine spread on distilled water, 25°C. Two experiments are shown, one at initial pressure, $\pi_i$, = 2 dynes/cm and the other at $\pi_i$ = 30 dynes/cm. Right panel: kinetic curves of surface potential at different values of initial pressure $\pi_i$ after injection of $CaCl_2$ 75 mM (final concentration) under films of dipalmitoyl phosphatidylcholine containing 10 and 50% acidic phospholipid dicetylphosphate, 25°C.

of the lipid onto the electrolyte solution, whereas such ion pairs cannot form and are not able to penetrate the interface when this was covered by the preformed phosphatidylcholine film [15,16]. Surface radioactivity and infrared spectroscopy should provide information about the chemical nature of the species penetrating the interface.

*Surface radioactivity and infrared spectroscopy*

It occurred to us that, in the absence of lipid, the adsorption of $^{45}Ca^{2+}$ to the air/water interface of $CaCl_2$ solutions increased with pH, quite dramatically between 9 & 10. In Table I we present such data for the pH values 4, 5.6, 9 and 10. The solutions contained 150 mM NaCl and 93 $\mu$M $CaCl_2$ as the $^{45}Ca^{2+}$ carrier. Upon spreading dipalmitoyl phosphatidylcholine to a film pressure of 30 dyne/cm, radioactivity and, thus $Ca^{2+}$ sorption, increased at low pH and decreased at high pH, markedly at pH 10. Desorption of $Ca^{2+}$ from the phosphatidylcholine films at high pH has been reported by some [5,17] but not by others [18].

The infrared spectra of films of $CaCl_2$ at pH 5.6 showed 3 distinct peaks at the frequencies 2.85, 4.65 and 6.15 $\mu$m (Fig. 4); we attribute these absorption peaks to the O-H stretching of $Ca(OH)_2$, since the magnitude of the peaks increased with both $CaCl_2$ concentration and pH. With $CaCl_2$, the three peaks were nil at pH 1 and huge at pH 10.

A model consistent with this observation is one in which $CaCl_2$ in water undergoes a reversible hydrolysis reaction

$$CaCl_2 + 2\,H_2O \xrightleftharpoons[H^+]{^-OH} Ca(OH)_2 + 2\,HCl$$

which is driven to the right by alkali and to the left by acid [16].

When dipalmitoyl phosphatidylcholine was spread on the $CaCl_2$ solution at pH 5.6 adsorption of $^{45}Ca^{2+}$ increased (Table I) and so did the intensities of the infrared absorption due to $Ca(OH)_2$. In other words, dipalmitoyl phosphatidylcholine caused more $Ca(OH)_2$ to be formed in the film and the infrared absorption to assume the characteristics of $CaCl_2$ at pH 10, as indicated by the split of the 6.15 $\mu$m peak (Fig. 6), which is typical of $Ca(OH)_2$ at pH 10. However, when dipalmitoyl phosphatidylcholine was spread on 75 mM $CaCl_2$ that was

TABLE I

INFLUENCE OF pH OF AQUEOUS PHASE ON THE RADIOACTIVITY IN cpm, OF CLEAN (CONTROL) AND DIPALMITOYL PHOSPHATIDYLCHOLINE FILM-COVERED SURFACE

The effect of the lipid film on $Ca^{2+}$ adsorption (+), or desorption (—), is shown in the column to the right. Subphase: 150 mM NaCl, 93 $\mu$M $CaCl_2$, 25°C; $^{45}Ca$, 0.27 $\mu$Ci/ml. The above values are averages of 3 separate experiments, with standard deviations <±20 cpm.

| pH | Radioactivity (cpm) | | |
|---|---|---|---|
| | Control | + Dipalmitoyl phosphatidylcholine | Δ (cpm) |
| 4 | 780 | 840 | +60 |
| 5.6 | 864 | 980 | +120 |
| 9 | 900 | 890 | −10 |
| 10 | 1200 | 990 | −210 |

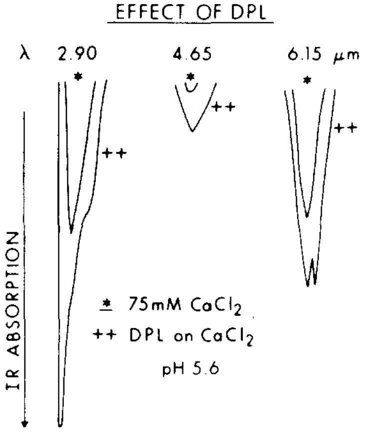

Fig. 4. Infrared (IR) absorption spectra of $CaCl_2$, pH 5.6, and saturated dipalmitoyl phosphatidylcholine (DPL) film spread onto the same $CaCl_2$ solution, 25°C. The peaks derive from O-H stretching at frequencies 2.85, 4.65 and 6.15 μm. The last two frequencies were characteristic of $Ca(OH)_2$, whereas the peak at or around 2.85 μm and peaks different from the 4.65 and 6.15 μm were produced by other electrolytes, such as uranyl nitrate, and long chain amines.

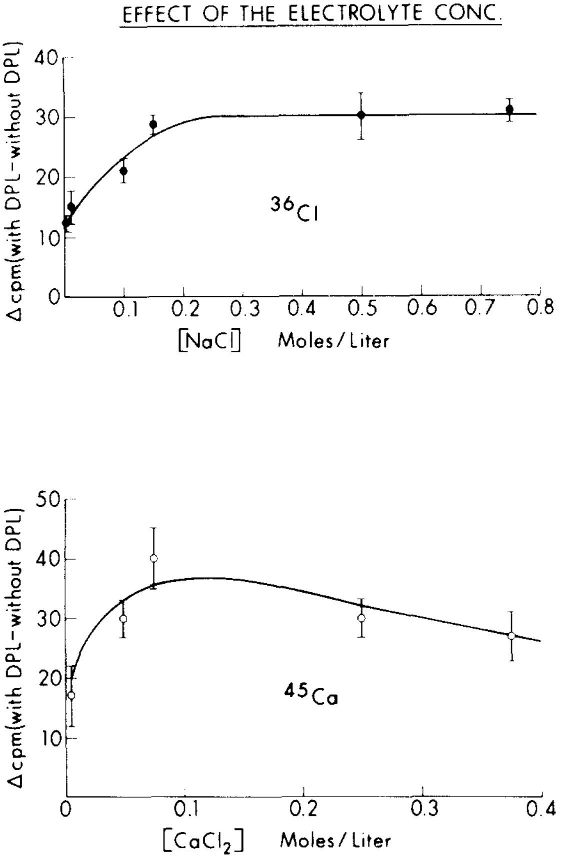

Fig. 5. Effect of ionic strength on the adsorption of $^{45}Ca$ and $^{36}Cl$ onto saturated films of dipalmitoyl phosphatidylcholine (DPL) spread on $CaCl_2$ and NaCl solutions, 25°C.

brought to pH 10 with NaOH, the surface radioactivity decreased markedly and so did also the infrared absorption typical of $Ca(OH)_2$. It remains to be established whether species other than $Ca(OH)_2$, such as $^+Ca(OH)$ and $^+CaCl$ are also present at the interface. The species connected with the hydrolysis reaction of $CaCl_2$ reveal the complexity of the ionic structure of the interface, and suggest that, in the absence of acidic phospholipid, correlations between surface potentials and interfacial dipoles remain to be defined.

*Effect of the ionic strength on $Ca^{2+}$ and $Cl^-$ adsorption.* We observed that the adsorption of $Ca^{2+}$ and $Cl^-$ onto dipalmitoyl phosphatidylcholine films at pH 5.6 increased with the concentration of the non-radioactive electrolyte, and it peaked or levelled off near 150 mequiv. NaCl or $CaCl_2$ (Fig. 5). Since other investigators worked with low $CaCl_2$ concentrations, <1 mM [5,17,18], some differences among various laboratories are explainable. Specifically, with the same quantity of radioactive ion ($Ca^{2+}$) in 93 μM $CaCl_2$ carrier in the subphase at pH 5.6, addition of dipalmitoyl phosphatidylcholine to the surface caused adsorption of 120 cpm in the presence of 150 M NaCl (Table I) but a desorption of 300 cpm of $^{45}Ca^{2+}$ in the absence of NaCl. This effect could explain the desorption of $Ca^{2+}$ observed by Hauser and Phillips at low $CaCl_2$ concentrations [5]. Since the adsorption of $Cl^-$ was also enhanced by the high NaCl concentrations (Fig. 5, upper panel), the adsorption of $Ca^{2+}$, $^-OH$, and $Cl^-$ ions speak in favor of the ion pair penetration, irrespective of whether $Ca^{2+}$ binds [5,6,19,20] or does not bind [7,21] with the phosphate group of phosphatidylcholine. The ionic strength effect bears also on the significance of the $Ca^{2+}$/phospholipid molar ratio. This was 0.54 in experiments with films of phosphatidylserine and, of course, much less (negative) with films of egg lecithin spread onto $10^{-4}$ M $CaCl_2$ [5]. According to the numerical interpretations of our surface radioactivity data with 150 mequiv. $CaCl_2$ or NaCl in the aqueous phase at pH 5.6, the $Ca^{2+}$/dipalmitoyl phosphatidylcholine ratio in the film varied between 1.5 and 2.5 [21]; the large excess of $Ca^{2+}$ and $Cl^-$ in the lipid film means electrolyte or ion pair penetration.

### *Speculations and biological significance*

The surface radioactivity measurements finally made another revelation. In the same system as described in Table I and Fig. 4, the quantity of $Cl^-$ adsorbed at pH 10 in the absence as well as in the presence of dipalmitoyl phosphatidylcholine was practically nil, whereas at pH 5.6, 10 to 40 $Cl^-$ and 2 $Ca^{2+}$ were adsorbed for each lipid molecule. The desorption of $Cl^-$ at high pH and the large excess of $Cl^-$ (and thus HCl) in the dipalmitoyl phosphatidylcholine film speak in favor of the hydrolysis reaction and mean that (a) in the absence of this lipid, the HCl leaves while the $Ca(OH)_2$ accumulates at the interface, and (b) in the presence of dipalmitoyl phosphatidylcholine, both $Ca(OH)_2$ and HCl accumulate at the interface, albeit necessarily in separate compartments, which may be provided by the specific interfacial architecture of phosphatidylcholine films. Should separate pools of electrolyte occur in the form of compartments in biological membranes, it is certainly a new phenomenon to be considered in studies of membrane functions.

With regard to whether the rise in transition temperature caused by divalent cations in monomolecular [22] and bimolecular [6,22,23] phosphatidylcholine membranes is due to electrostatic interaction of the salt's cation with the phosphate group of the lipid, we suggest that such an interaction is not necessary. Instead, penetration of $Ca^{2+}$, $Cl^-$, $Ca(OH)_2$, $H^+$ and $^-OH$ in the lipid film would bring about formation of a high viscosity interfacial lattice [24,25] whose collapse (preceding the melting of the alkyl chains) must require a higher temperature. The effects that these hydrophilic lattices may have on the NMR effects of the protons of the phosphorylcholine group of phosphatidylcholine [5,19,20] remain to be investigated and eventually separated from effects of direct ion-lipid interaction. Meanwhile, any attempt at extricating the surface potential dipoles from the maze of such lattices must await the results of systematic experimentations involving (a) the molecular correlates of surface potential, (b) direct spectroscopy of the air/water and lipid/water interfaces and (c) the identification of the interfacial ionic species of water, electrolyte and lipid.

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