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LIPID MONOLAYERS: SURFACE POTENTIAL OF DIPALMITOYLLECITHIN WITH REGARD TO ION SORPTION AND Ca^{2+} BINDING

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SUMMARY

In line with previous work, the surface potential of commercial preparations of dipalmitoyllecithin on aqueous support increased in the order water $<$ NaCl $<$ $CaCl_2$. This effect, indicating counterion binding on acidic sites, was not observed, however, after purification of lecithin on a silicic acid column.

The potentials that previous authors attributed to fixed dipoles of the lipid do not exist. The increased potentials of dipalmitoyllecithin on $CaCl_2$ solutions in comparison with NaCl were the effect of interaction of Ca^{2+} with the fixed charges of acidic contaminants present in ordinary preparations of the otherwise neutral phospholipid.

The electrical potential of the Gouy diffuse double layer found in monolayers of the neutral phospholipid is indeed produced by ionic impurities. The claim that the fixed dipoles have large potentials comparable to those of fixed charges is thus incorrect. The fallacy is caused by a fundamental error in the theory of surface potentials at the point where the formalism of the parallel plate capacitor, requiring free charges, is applied to the treatment of uncharged dipoles, which are systems of neutralized charges.

INTRODUCTION

The transport of ions across biological membranes and the latter's participation in electrical activity have been for obvious reasons associated with the presence of fixed charges on the biological surfaces. These could then function as ion exchangers and carriers. The role of the acidic phospholipids in these processes is obvious. However, the widespread occurrence of phosphatidylcholine (lecithins) in biological membranes has long raised the question as to whether the phosphorylcholine group of this lipid participates in ion binding. Both the experimental data and views on this question have been widely divided.

Experimental background

After surface-potential measurements, Anderson and Pethica[1] suggested binding of cations from various electrolyte solutions on monolayers of synthetic distearoyllecithin, and Shimojo and Ohnishi[2] concluded that egg lecithin and sphingomyelin monolayers bound Ca^{2+} from solutions containing 0.5 mM $CaCl_2$. Kimizuka and Koketsu[3] demonstrated adsorption of $^{45}Ca^{2+}$ on monolayers of dipalmitoyllecithin at an area

of 60 Å^2 per molecule, whereas Santis and Rojas[4] found no adsorption of radioactive Ca^{2+} on monolayers of dipalmitoyllecithin from solutions containing 2 mM $CaCl_2$. Similarly, Hauser and Dawson[5], using radioactive Ca^{2+} showed that monolayers of egg lecithin did not bind Ca^{2+} and suggested that ionic impurities could be responsible for any such binding by the otherwise neutral phospholipid. In contrast, Shah and Schulman[6] and later Vilallonga *et al.*[7] used surface potential measurements and concluded that monolayers of synthetic dipalmitoyllecithin interacted with Ca^{2+}.

A clue to the confusion came from experiments at the oil–water interface[8], where chromatographically homogeneous egg lecithin having a zero net charge exhibited no interaction with Ca^{2+}. Less homogeneous preparations of synthetic phosphatidylethanolamine and phosphatidylcholine and animal cephalin, which interacted with Ca^{2+}, contained variable quantities of acidic impurities. Apparently, that report was ignored by subsequent investigators, who mistook interaction of Ca^{2+} with acidic contaminants for interaction of Ca^{2+} with molecular dipoles[6,7]. In spite of the suggestion of Hauser and Dawson[5] and Colacicco[8], Shah and Schulman[9] continued to explain that Ca^{2+} binds on certain dipoles of the phosphate group of phosphatidylcholine (lecithin) and this interaction causes the increase in surface potential which is observed on $CaCl_2$ as compared with NaCl. Various ionic effects were also observed at the air–water interface with various lipid preparations (sphingomyelin, lecithin and cephalin) that contained ionic contaminants[10]. Once the lipids were purified, the ionic potentials disappeared, but they returned when either the contaminants were added back or the lipids were stored under unsuitable conditions.

It became apparent that the different, and at time conflicting, effects reported be several investigators can now be explained by and traced to experimental conditions and especially the quality of the lipid preparations[10]. However, since much of the confusion is caused by a want of knowledge in the theory of surface potentials, it is to both questions that the present communication is addressed. The experiments with dipalmitoyllecithin were prompted by a report in which this lipid was used as a model for calculations and theories[11] which proved in support of erroneous observations[6,9].

Theoretical considerations

Calculations of the potential profile of a model lecithin membrane at 25 °C were performed by Gillespie[11], who claimed that the potentials due to fixed dipoles are large and are comparable with the electrical potentials of fixed charges. That is wrong. The error in the theory stems from the formalism of surface potentials. Experimentally[12,13], the interface is placed between an air electrode (+) and a water electrode (−), calomel or ground, connected with a high impedance electrometer. Operationally, the surface potential ΔV is the difference between two electrometer readings, one for the surface covered with film (V) and one without the film (V_0). Conceptually, ΔV has been made to consist of two contributions[6,12]. For given values of dielectric constant D and surface concentration of molecules $\bar{n}/A$, one contribution, $4\pi\bar{n}\mu/DA$, referred to as the Helmholtz potential, is attributed to the vertical component (μ) of surface dipoles of the uncharged species[6,9]. The other contribution, $\Delta\psi$, is referred to as the Gouy potential or potential of the fixed charges. Discussion of both contributions is necessary to shed some light on the theoretical treatment of Gillespie[11] and the variety of results obtained in different laboratories.

The electrical potential of the ionized groups on the surface of the membrane is

pretty well understood. The picture is that of a Gouy's diffuse double layer[12], in which an hydrophilic species stretches the ionic group out of the interface of the membrane and thus shows a net negative or positive charge. The magnitude of this potential depends on the surface concentration of fixed charges and on the concentration and charge of counterions in the aqueous phase; the ionic potential of the fixed charge vanishes when enough counterions neutralize the ionized species. Such an ionic potential is similar if not identical with the electrokinetic potential and with the only electrical potential that one measures with saturated calomel electrodes at the oil–water interface[8]. The physical meaning of the measurements is such that the appearance of a negative or a positive charge at the water interface (negative pole of the electrometer) causes respectively a negative or positive change in potential. This part of the surface potential obviously applies to the ionic lipids.

The contribution attributed to the surface dipoles of the uncharged species is controversial[10,14]. The electrical potential across a mono-layer of neutral lipids, $\Delta V = 4\pi\bar{n}\mu/DA$, has been modeled after the potential of a parallel plate capacitor; in this, ΔV is $4\pi\bar{n}(ed)/DA$. The physical reality of the product (ed), equivalent to charge x distance between plates, has the dimensions of the dipole moment but in effect expresses a separation of charges. The only system that this equation describes is the same one treated by the Gouy potential of the diffuse double layer[15], $\Delta\psi = (4\pi/D)\ (\sigma/AK) = (4\pi/D)(\bar{n}/A)el$. There, σ is the total charge and $1/K$ is the Debye length or l, namely the thickness of the Helmholtz double layer[15]. Although the surface chemists have long claimed that the Helmholtz equation handles dipoles[6,9,11,12] in effect it describes charges, which belong to ionized surface water[16] and probably not to the lipid. The discrepancy causes a fundamental error, which was not challenged heretofore and becomes apparent during the analysis of Gillespie's treatment[11]. On this ground, both the "Helmholtz" equation $\Delta V = 4\pi\bar{n}\mu/DA$ used by Shah and Schulman[6,9] and Gillespie's treatment of dipoles[11] are unacceptable, and can be rejected at once. However, experimentation with dipalmitoyllecithin can be used to confirm the thesis. Previous objections to the Helmholtz equation were such that, in the absence of either an alternative[16] or an explanation, the best course was to use the equation as it was[14].

MATERIALS AND METHODS

Lipids

Preparations of synthetic dipalmitoyllecithin were purchased from Mann Research Laboratories, New York. Other preparations of dipalmitoyllecithin were obtained from Applied Science Laboratory, State College, Pa. Synthetic dimyristoyllecithin was purchased from LaMotte Chemical Company, Chestertown, Md. Other lipid preparations were described[17,18]. Depending on the quality of the preparation, either the contaminants were conspicuous on thin-layer chromatography plates even when a minimal quantity of (100 μg) lipid was applied, or large quantities of lipid, 500–1000 μg, were required to show the contaminants. The chromatography used glass plates coated with silica gel H (Merck, Darmstadt, Germany) and solvent mixtures containing chloroform–methanol–water–conc. [NH_4]OH (70:30:4:1, by vol.)

Column chromatography

Improvement of lecithin preparations was effected by chromatography on silicic acid columns at room temparature (25 °C). A slurry of 10 g Unisil (200–325 mesh silicic acid) with 25 ml chloroform was poured in a column of pyrex glass, 25 cm in length and 1 cm in internal diameter. The column was washed with 100 ml chloroform. The sample, 100 mg dipalmitoyllecithin was applied in 2 ml chloroform. The column was eluted successively with 100 ml each of the following chloroform–methanol mistures, 100:0, 90:10, 80:20, 70:30, 60:40, 50:50 (by vol.), and finally methanol. The fractions were monitored by thin-layer chromatography.

Measurements at the air–water interface

Force–area and surface potential–area curves were obtained both in the rectangular trough[13] and in the circular, constant area, trough[19]. Surface pressure was determined after measurement of surface tension by a sandblasted platinum blade suspended from a torsion balance; surface potential was measured by a radioactive (^{226}Ra) air electrode[13]. Trough and barrier were coated with purified paraffin, m.p. 48 °C (Fisher Scientific, New York). The methods for the preparation of water, solvents, lipids solution and films have been described[13].

RESULTS

A typical picture of the surface potential of dipalmitoyllecithin is summarized in Fig. 1. For all practical purposes, the force–area curve, lower panel, was the same, on either water, 0.01 M NaCl or 0.01 M $CaCl_2$, before and after purification of the lipid. In contrast the surface potential values (upper panel) were influenced appreciably by improvement of the dipalmitoyllecithin preparation. The surface potential of purified dipalmitoyllecithin was the same (curve C) on water, NaCl or $CaCl_2$ at concentrations between 0.1 mM and 0.1 M. Before purification on silicic acid column, dipalmitoyllecithin on 0.01 M NaCl (Curve A) produced a surface potential which was 15 mV greater than that on water (not shown) and appreciably smaller than that on 0.01 M $CaCl_2$ (Curve B). After removal of the impurities, Curve C was obtained which was identical on water, 0.01 M NaCl or 0.01 M $CaCl_2$. Addition of the impurities to the purified preparation C restored Curve A, produced a lower curve on water (not shown) and the higher Curve B on 0.01 M $CaCl_2$.

The foregoing observations are confirmed and expanded in Fig. 2, where the surface potential values ΔV of dipalmitoyl lecithin at an area of 50 $Å^2$ per molecule are related to the log of the electrolyte concentration between 0.1 mM and 0.1 M. A typical preparation A gave a surface potential which was lowest on water and increased with increasing concentrations of NaCl. As in Fig. 1, the surface potential on $CaCl_2$, Curve B, was significantly higher than that on NaCl. Improvement of the lecithin preparation A by chromatography on silicic acid column yielded a preparation C which was free of acidic impurities: The surface potential (Curve C) was then independent of electrolyte concentration and was the same on either water, NaCl or $CaCl_2$.

These phenomena were verified by the electrical potentials at the oil–water interface[8]. As expected, preparation A, which contained acidic contaminants, had a negative $\Delta\psi$ potential, indicating a negative charge. The magnitude of the negative deflection increased with (1) the concentration of anionic contaminants, and (2) the quantity of lipid applied on a given area up to saturation.

The impurities obtained from the silicic acid column were grouped in two main fractions (I and III), namely the chloroform and the methanol fractions (Fig. 3). The chloroform fraction, the first one out of the silicic acid column, showed lipid staining material migrating with the front on thin-layer plates. Occasionally, this fraction manifested a positive reaction with ninhydrin spray (Fig. 3, spot N). When applied either directly or after elution from thin-layer plates, this fraction showed a zero

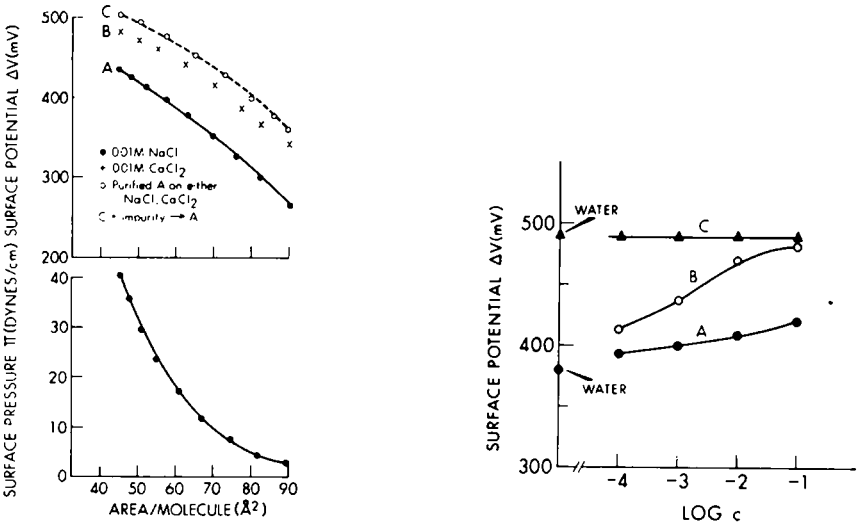

Fig. 1. Surface pressure–area and surface potential–area curves of dipalmitoyllecithin on various aqueous supports at 25 °C. The force–area curve was the same on the various subphases.

Fig. 2. Influence of electrolyte concentration and quality of lipid on the surface potential of dipalmitoyllecithin at an area of 50 Å²/molecule. A, commercial preparation of dipalmitoyllecithin on NaCl; B, same on $CaCl_2$; C, purified dipalmitoyllecithin on either water, NaCl, or $CaCl_2$. Temperature 25 °C.

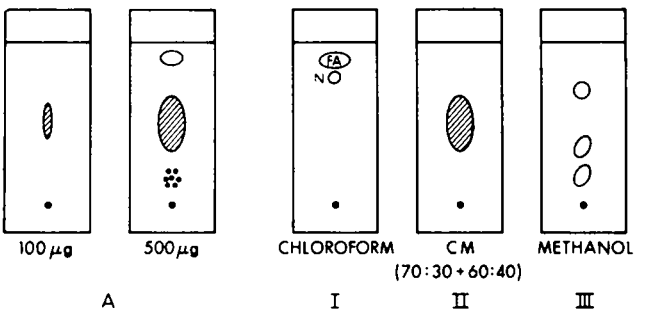

Fig. 3. Schematic representation of thin-layer chromatography plates of (A) the commercial preparation of dipalmitoyllecithin and Fractions I, II and III after elution from the silicic acid column. The impurities, which did not show when 100 μg phospholipid was applied, were evident with 500 μg or larger applications. The impurities were conspicuous after isolation. Fraction I: N, long chain amine; FA, fatty acid. Fraction II; purified dipalmitoyllecithin. Fraction III: acidic impurities; all three acid impurities, shown in this preparation, contained phosphorus. The quantity of phosphorus in Fraction III varied between 2 and 5% of the total phosphorus in the various preparations which produced results similar to the ones shown in Figs 1–3. As much phosphorus, however, was found also in the form of unidentified neutral and acidic impurities that were eluted with the chloroform–methanol (CM) (90:10 and 80:20 by vol.) fractions.

charge or a slightly positive charge on water as well as on 1 mM NaCl at the oil–water interface. The positive charge was consistent with the ninhydrin reaction of this fraction. The methanol fraction, instead, was strongly acidic and displayed a negative charge on either water or 1 mM NaCl. Another group of impurities, neutral and acidic, were found in the elution fractions containing chloroform–methanol (90:10 and 80:20, by vol.). (See legend of Fig. 3.)

The trends shown in Figs 1–3 were reproduced with numerous preparations of lecithins, such as yeast and egg lecithins, phosphatidylcholine from beef heart, phosphatidylethanolamine (cephalin) from egg and phosphatidylethanolamine from beef brain, synthetic dimyristoyllecithin and hydrogenated egg lecithin. Addition of acidic lipid contaminants to the purified phospholipids produced preparations which displayed a Ca^{2+} effect. The magnitude of the effects varied with the distribution and concentration of impurities; and the correlations were clear and consistent.

DISCUSSION

An increase in the surface potential values of commercial preparations of phosphatidylcholine on $CaCl_2$ in comparison with NaCl has been interpreted to mean interaction of the lipid with Ca^{2+}. The mode of interaction, however, has been controversial[6,9,10].

Under no circumstances did purified dipalmitoyllecithin produce a surface potential that was sensitive to Ca^{2+}. Since the pH of water, 0.01 M NaCl and 0.01 M $CaCl_2$ solutions was about the same, 5.7, the data confirm a known fact, namely that between pH 4 and 9 phosphatidylcholine at the air–water interface is a neutral phospholipid[20,21].

Mechanisms of surface potential changes

Whereas Gillespie's[11] theory, in line with the suggestion of Shah and Schulman[6], claims a treatment of the dipolar properties of the phosphate group of the phospholipid, the experimental results reflect only the ionic properties of the acidic contaminants. The fact that these effects disappear after removal of the acidic impurities indicates that ion sorption in general and Ca^{2+} interaction in particular are not due to the dipolar interactions predicated by the other investigators. The foregoing data indicate also that the theoretical treatment of the presumed molecular dipoles made by Shah and Schulman[6,9] and Gillespie[11] gave Gouy potentials which cannot be verified experimentally with purified lecithins at 25 °C and which belong to ionic impurities. Should such ion–dipole interactions exist, they do not affect the surface potentials. That not withstanding, the salt linkage or ion pair of the phosphorylcholine group of lecithin can open up under the action of either high electrolyte concentrations ($>$ 1 M NaCl, $>$ 0.3 M $CaCl_2$, unpublished data), extreme pH values[20,21], long chains ions[22], or higher temperatures[23] such as 37 °C. A similar ion-exchange mechanism was proposed by Anderson and Pethica[1] for the interaction of distearoyllecithin with electrolytes; such interactions, however, cannot be verified with 0.01 M NaCl or $CaCl_2$. In any such case[1,20–23], the phosphorylcholine ion pairs must be dealt with as systems of free charges in ion-exchange processes and not as dipoles.

Unfounded also are the claims that dipole moments calculated from surface potential measurements can be used to determine the orientation of the phosphoryl-

choline group of lecithin[6,24]. Since the effects attributed to dipoles[6,11] did not exist, the vertical orientation proposed by Shah and Schulman[6,9] is untenable.

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