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A NEW METHOD FOR INVESTIGATION OF LIPID ASSEMBLIES WITH A LIPOID pH INDICATOR IN MONOMOLECULAR FILMS*

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SUMMARY

The lipoid pH indicator 4-heptadecylumbelliferone is embedded in monomolecular lipid films. The influence of different lipid mixtures, which are related to lecithin, on the proton equilibrium of the indicator was studied. Local pK and pH modulations were found. The modification of the monolayer technique used for these investigations is described.

INTRODUCTION

The properties of a lipid molecule in a lipid bilayer or monolayer may depend on the presence of other lipid molecules. A lipid mixture may have properties not found with the pure lipid components. By appropriate combination of different lipids, new properties may by obtained. Such an aggregate of cooperative lipid molecules may be called a "lipid assembly". Several examples of such lipid assemblies are studied with monolayers at the air–water interface. As a probe to characterize certain properties of these lipid assemblies a lipoid pH indicator is added in a small concentration. The shift of the apparent pK of the indicator is observed as the lipid composition of the film and the ion content of the subphase are changed.

First the variant of the monolayer techniques is described which has been developed for these investigations. Then some results are presented demonstrating the implications of the lipoid pH indicator method as applied to lipid assemblies related to lecithin.

MATERIALS AND METHODS

Fig. 1 shows the procedure of preparing the lipid film sample. A monomolecular film is made by spreading a lipid solution in chloroform onto the water surface of a monolayer trough. The film is held under a constant surface pressure (25–45 dynes/cm) by an automatic device[1]. A hydrophobic glass slide (cleaned ultrasonically, treated with dimethyldichlorsilane and covered with two layers of methylstearate)[2] is slowly dipped through the film. The slide, thus covered with a single lipid mono-

* This method has been presented at the Jahrestagung der Gesellschaft für Physikalische Biologie, Göttingen, Germany, May 1972.

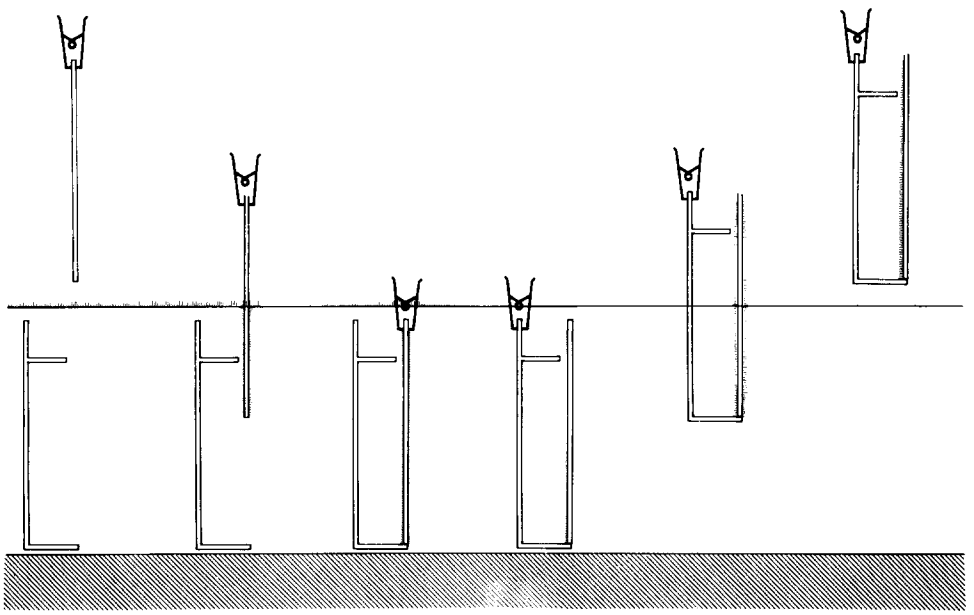
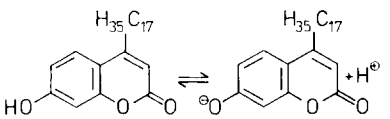


Fig. 1. Schematic drawing showing the procedure of preparing a monomolecular film to investigate its interactions with water solutions. The monolayer is transferred onto the slide, which at the same time assumes its function as the fourth wall of a cuvette standing below the water surface.

layer (the hydrophobic chains attached to the slide), fits precisely into the groove of a cuvette standing in the trough. Now the slide has assumed its function as the fourth wall of the cuvette. After removal of excess film the cuvette is taken out of the trough. The lipid monolayer is now on the inside of one wall of the cuvette. Two joints at the cuvette allow the solution which is in contact with this lipid film to be exchanged.



Formula I

To each lipid solution (concentration 5 mM) two percent (per lipid chain) of the indicator 4-heptadecylumbelliferone (Formula I)[3] are added. The monolayers investigated consist of methylpalmitate (Merck), octadecane (Merck), sodium octadecylsulfate (Schuchardt), eicosyltrimethylammoniumbromide (Schuchardt, twice recrystallised) and dipalmitoyllecithin (Koch-Light). Fig. 2 shows the position of the indicator within the lipid film. The sensitive chromophore is lying between the hydrophilic headgroups of the lipid molecules.

4-Heptadecylumbelliferone as well as umbelliferone exhibits a high fluorescence quantum yield in the dissociated state and a very low one in the undissociated state[4,5]. Thus by exciting the dye by light of 366 nm and measuring the fluorescence at 440 nm, while changing the bulk pH in the cuvette by stepwise exchange of the solution, the dissociation of the dye may be observed directly. (The pH of the solution is measured with a glass electrode relative to NBS buffer solutions). In order to com-

pensate for changes in quantum yield of the dye anion in different monolayers the fluorescence signals are divided by the signals measured for complete dissociation at high pH (pH 11 or 12) for each lipid film separately. Thus calibrated titration curves are obtained.

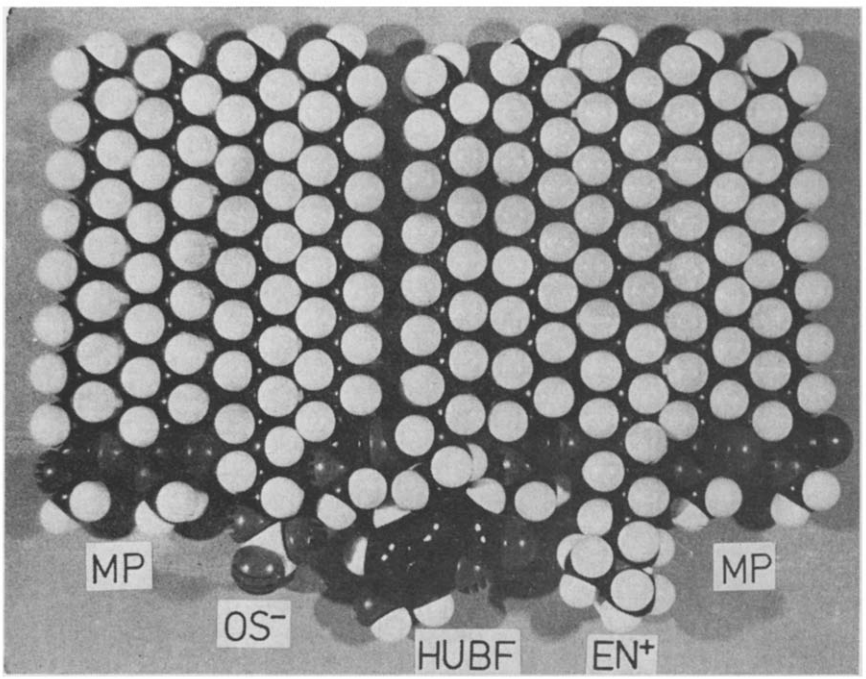


Fig. 2. Molecule models of a methylpalmitate film (MP) with one octadecylsulfate (OS^-) and one eicosyltrimethylammonium (EN^+) model showing the position of the 4-heptadecylumbelliferone (HUBF) in the lipid film. The sensitive indicator group lies in the plane of the polar head groups of the lipids.

RESULTS

In Fig. 3 the titration curves of 4-heptadecylumbelliferone in a dipalmitoyllecithin–octadecane 1:1 film (Lec–O) are compared with 4-heptadecylumbelliferone in a methylpalmitate film (MP) with NaCl and $CaCl_2$ solutions both at an ionic strength of 10 mM. In both films the apparent pK of 4-heptadecylumbelliferone is shifted to higher values relative to umbelliferone in solution (pK 7.75[5]). Moreover in lecithin–octadecane the pK is 0.7 unit higher than in the methylpalmitate film. The presence of Ca^{2+} in both cases causes a shift of the apparent pK to lower values, *i.e.* a higher degree of dissociation. In lecithin–octadecane this shift is nearly twice that of the shift in methylpalmitate. ($\Delta pK = 1.0$ and 0.6 resp., Fig. 3).

Now a lipid assembly is designed consisting of methylpalmitate and octadecylsulfate of a ratio of 2:1. This assembly closely resembles lecithin–octadecane without the choline group, the singly charged phosphate group being replaced by the isoelectronic sulfate group. (*cf.* Fig. 4). Fig. 5 shows that in this film (MP–OS^-) the pK

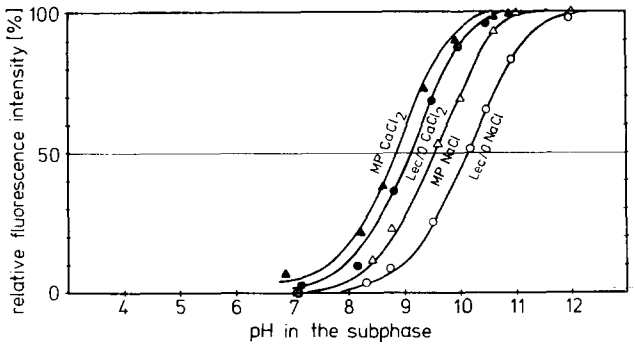


Fig. 3. Relative fluorescence intensity of 4-heptadecylumbelliferone in different lipid films *versus* pH of the subphase for NaCl and $CaCl_2$ solutions both with an ionic strength of 10 mM. The figure shows the results for methylpalmitate (MP) and dipalmitoyllecithin–octadecane, 1:1 (Lec–O).

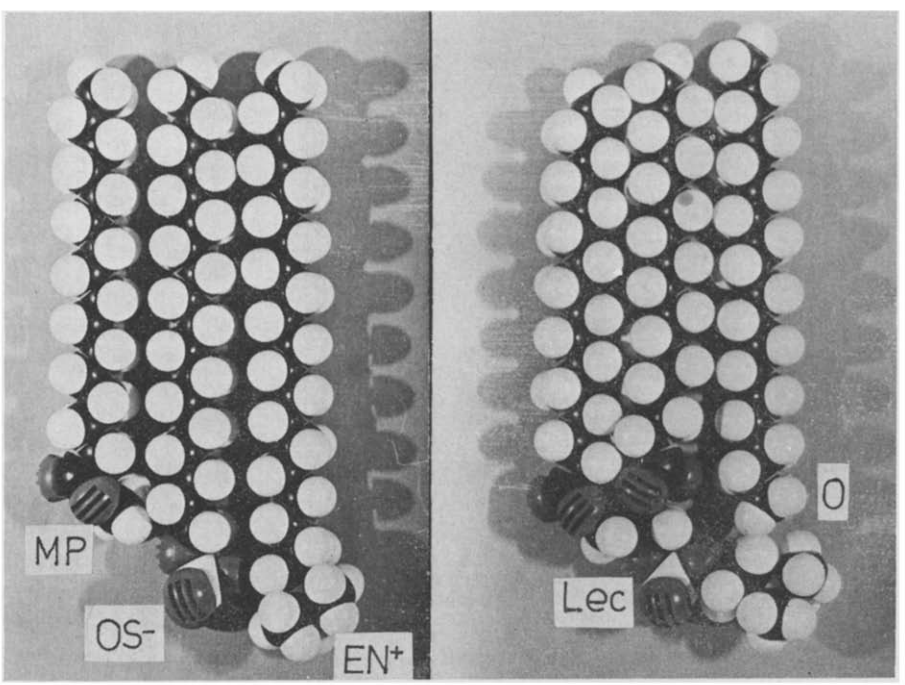


Fig. 4. Molecule models of methylpalmitate–octadecylsulfate–eicosyltrimethylammonium, 1:1:1, (MP–OS^-–EN^+, left) and dipalmitoyllecithin–octadecane (Lec–O, right). The models demonstrate how lecithin, in the conformation indicated, is approximated by a lipid assembly consisting of three different molecules (MP, OS^-, EN^+).

of 4-heptadecylumbelliferone is 0.9 unit higher than in lecithin–octadecane. In the MP/OS^- film Ca^{2+} causes a larger shift than in the Lec/O film (Δ pK is 1.5 compared to 1.0).

The negative sulfate group may be neutralized by an eicosyltrimethyl ammonium ion in a methylpalmitate–octadecylsulfate–eicosyltrimethylammonium 1:1:1 film

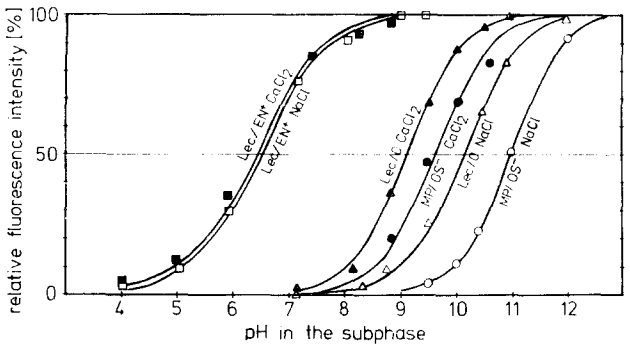


Fig. 5. Relative fluorescence intensity of 4-heptadecylumbelliferone in different lipid films *versus* pH of the subphase for NaCl and $CaCl_2$ solutions both at an ionic strength of 10 mM. The figure shows the results for dipalmitoyllecithin–octadecane, 1:1 (Lec–O), methylpalmitate–octadecylsulfate, 2:1 (MP–OS^-) and dipalmitoyllecithin–eicosyltrimethylammonium, 1:1 (Lec–EN^+).

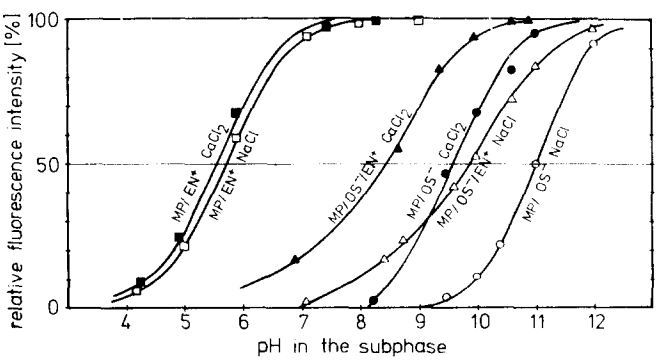


Fig. 6. Relative fluorescence intensity of 4-heptadecylumbelliferone in different lipid films *versus* pH of the subphase for NaCl and $CaCl_2$ solutions both at an ionic strength of 10 mM. The figure shows the results for methylpalmitate–octadecylsulfate, 2:1 (MP–OS^-), methylpalmitate–octadecylsulfate–eicocyltrimethylammonium, 1:1:1 (MP–OS^-–EN^+) and methylpalmitate–eicosyltrimethylammonium, 2:1 (MP–EN^+).

(MP–OS^-–EN^+). Thus a lipid assembly is constructed having a structure similar to that of lecithin–octadecane (see Fig. 4). In Fig. 6 it is shown that the large pK shift in the methylpalmitate–octadecylsulfate (MP–OS^-) is compensated in the methylpalmitate–octadecylsulfate eicosyltrimethylammonium film resulting in a pK of 9.9 which is close to lecithin–octadecane (Fig. 5, Lec–O, pK = 10.1). The effect of Ca^{2+} in the overall neutral methylpalmitate–octadecylsulfate–eicosyltrimethylammonium film (Fig. 6, MP–OS^-–EN^+, ΔpK = 1.5) is considerably higher than in pure methylpalmitate (Fig. 3, MP) as found with lecithin–octadecane (Fig. 3, Lec–O).

For completeness in Fig. 6 also the results for the positive methylpalmitate–eicosyltrimethylammonium, 2:1 film (MP–EN^+) are depicted. A large shift to low pK and no Ca^{2+} effect are observed.

In Fig. 5 lecithin–octadecane furthermore is compared with an assembly where an additional positive charge is introduced by replacing the octadecane by

an eicosyltrimethylammonium ion (Lec–EN^+). It is observed that this external neutralization of the phosphate group by an adjacent fixed positive charge causes a large shift of apparent pK. With this overall positive assembly lecthin–eicosyltrimethylammonium no Ca^{2+} effect is observed at all.

All the titration curves demonstrate the change of the proton equilibrium of 4-heptadecylumbelliferone with changing bulk pH in monolayers of different lipid composition. If different lipid assemblies are placed on the same slide next to each other, different states of the dissociation equilibrium exist at the same time with the same bulk pH. Such a local modulation of apparent pK by local modulation of lipid composition is demonstrated in Fig. 7 with cholesterol, lecithin and cholesterol–lecithin, 1:1. The slide is scanned at four different pH values through the exiting light beam. At pH 12 in all three zones the 4-heptadecylumbelliferone is completely dissociated. At pH 10 in the cholesterol zone there is still a high degree of dissociation whereas in the lecithin zone about half of the dye molecules are dissociated. (The

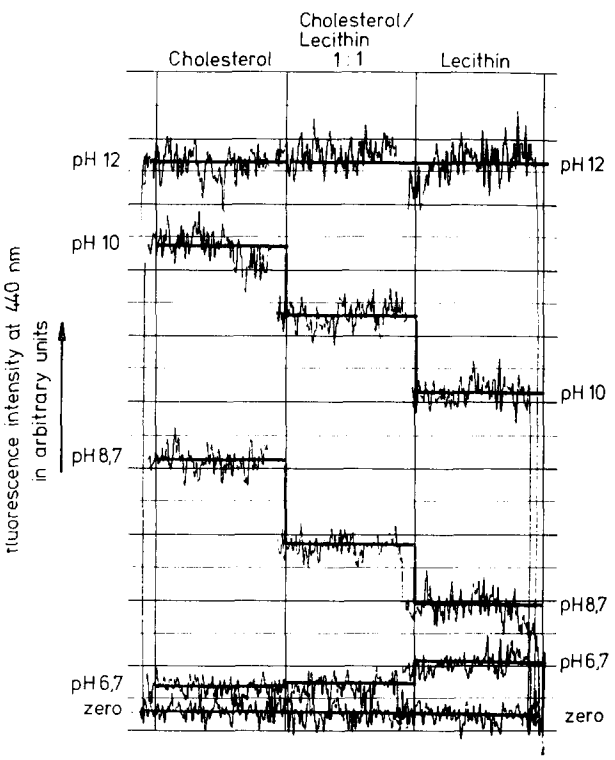


Fig. 7. Fluorescence intensity of 4-heptadecylumbelliferone in monolayers of cholesterol, cholesterol–dipalmitoyllecithin, 1:1, and dipalmitoyllecithin at different pH values of the subphase. The slide covered with three zones of these three lipid films is scanned at four different pH values of the subphase across the exciting light beam. The resulting fluorescence signal is registered continuously. An appropriate mean value is indicated. (The signal in the three zones is calibrated by change of amplification to give at pH 12 the same deflection (amplification factors at all pH values 1.00, 1.05 and 1.2 from left to right). The occurrence of different states of the proton equilibrium in different parts of the lipid layer at the same time with the same subphase is seen directly.

apparent pK of 4-heptadecylumbelliferone in cholesterol is approx. 9.0 and in lecithin approx. 10.0 as obtained by measuring the complete titration curve.)

DISCUSSION

Measurement of the relative fluorescence intensities gives direct information on the fraction of dissociated 4-heptadecylumbelliferone molecules. This fraction may be changed by a change of proton concentration at the location of the indicator molecules or by a change of the pK of the proton equilibrium due to the surroundings.

A change of the pK may be caused by a change of the dielectric constant or by fixed charges which stabilize or destabilize adjacent indicator anions. A change of local proton concentration is to be expected with an electrically charged interface due to the existence of an electrical diffuse double layer.

In the lipid films without any fixed charges, the 4-heptadecylumbelliferone shows apparent pK values which are shifted to higher values relative to umbelliferone in solution (cholesterol Δ pK=1.2, methylpalmitate Δ pK=1.7 in NaCl solution (Figs 3 and 7)). Neglecting the influence of the heptadecyl substituent the shift may be assigned to a lower dielectric constant near the lipid film (when compared to measurements with *p*-nitrophenol in water–methanol mixtures[6,7] these pK shifts of umbelliferone correspond to dielectric constants of 45 and 38, respectively). The shift might also be caused by a negative charging of the film by adsorbed anions. Further investigations should clarify the point.

The large shift in apparent pK in methylpalmitate–eicosyltrimethylammonium (Fig. 6) can be explained by the existence of the diffuse electrical double-layer resulting in decreased proton concentration at the lipid film. Using the Gouy theory[8] a pH shift of 3.3 is obtained for the charge density of this film. This agrees fairly well with the experimental shift of the apparent pK of 3.7 (Figs 3 and 4). In contrast to this positive lipid mixture the negative methylpalmitate–octadecylsulfate film with the same charge density shows a pK shift in NaCl solution of 1.5 (Fig. 6, MP–OS^-) which is far less than the theoretical value. Possibly in this case, Na^+ are adsorbed to the sulfate groups thus reducing the negative charge density. (A detailed study of electrically charged monolayers will be published elsewere). (Fromherz, P, and Masters, B.).

With lecithin–octadecane large shifts due to local pH changes do not occur since the fixed positive and negative charges of the phosphatidylcholine group compensate each other. The remaining destabilization of the indicator anion relative to methylpalmitate (Fig. 3) may be due to the direct influence of the phosphate group. Also a different dielectric constant is conceivable.

The lipid assembly methylpalmitate–octadecylsulfate–eicosyltrimethylammonium resembling lecithin–octadecane (Fig. 4) shows internal compensation of the pH shift also. Similar to lecithin–octadecane also in the overall neutral lipid assembly the apparent pK of 4-heptadecylumbelliferone is higher than in methylpalmitate (Figs 3 and 6). (At lower concentations of the positive and negative charges, *e.g.* methylpalmitate–octadecylsulfate–eicosyltrimethylammonium, 10:1:1, the apparent pK is the same as with pure methylpalmitate.) The titration curve is somewhat less inclined than in all other cases. This may indicate that not all the dye molecules are in exactly the same microenvironment with respect to the charges.

As to the effect of Ca^{2+} (relative to Na^+) on the apparent pK of 4-heptadecylumbelliferone it is not decided whether this is due to the binding of Ca^{2+} to the lipids, the resulting higher positive or lower negative charge stabilizing the dye anion, or whether the Ca^{2+} binds directly to the indicator anion, the charged lipids supporting or impeding the stabilization of the dye anion by this Ca^{2+} binding.

With the unpolar methylpalmitate Ca^{2+} causes only a small shift (Fig. 3, MP). With methylpalmitate–octadecylsulfate the calcium binding is so effective that the influence of the negative charges is completely compensated and the apparent pK of the indicator in this case is nearly the same as with pure methylpalmitate (Figs 5 and 3).

In the lipid assembly methylpalmitate–octadecylsulfate–eicosyltrimethylammonium the calcium binding is not effective enough to give such a compensation of the influence of the negative sulfate groups (Fig. 6, MP–OS^-–EN^+, $CaCl_2$ and MP–EN^+, NaCl), since the fixed positive charges impede positive charging of the film by Ca^{2+}. However, Ca^{2+} shifts the apparent pK of 4-heptadecylumbelliferone with this overall neutral film much more than in the intrinsic neutral methylpalmitate, indicating a specific action of the negative groups within the neutral film.

Lecithin–octadecane again behaves similar to the methypalmitate–octadecyleicosyltrimethylammonium lipid assembly: the pK shift of 4-heptadecylumbelliferone caused by calcium is far more pronounced than with the intrinsic neutral methylpalmitate (Fig. 3), indicating a specific action of the negative phosphate groups. On the other hand the shift is too small to account for complete neutralization of the phosphate groups by Ca^{2+} (see *e.g.* ref. 9). This is seen by comparison of lecithin–octadecane, $CaCl_2$ with lecithin–eicosyltrimethylammonium (Fig. 5, Lec–O and Lec–EN^+): here the phosphate groups are neutralized by an additional fixed positive charge and the shift is found to be much more pronounced.

The changes of apparent pK found by adding cholesterol to lecithin (Fig. 7) may be due to the dilution of the phosphate groups and/or by a change of the dielectric constant near the lipid film.

The experiments described show how lipid assemblies may be designed to provide different conditions for proton equilibria at a lipid layer. The conditions may be modified by ions interacting with the lipid assembly. Local change of lipid composition may cause a local modulation of these conditions. The local modulation of the state of a proton equilibrium is modified by ions in the subphase.

Lipid assemblies as described not only provide new properties with respect to proton equilbria. They also have effects on enzymes adsorbed to the lipid monolayers, different from the effects of the pure lipid components (Peters, J. and Fromherz, P., unpublished). (Also spectroscopic properties of dyes may be modified by appropriate lipid mixtures[10]).

The elementary physical interactions giving rise to all these effects may be different with different lipids as shown with electrically charged and uncharged lipid assemblies.

More generally the experiments shown how new structures and functions of a lipid film may be attained by using an appropriate "lipid assembly". Obviously such new properties may be attained just as well by synthesizing an appropriate lipid compound. These two possibilities have been compared with the methylpalmitate–octadecylsulfate–eicosyltrimethylammonium film and the lecithin–octa-

decane film. By combining more complicated lipids to give a lipid assembly, structures and functions not attainable with covalent compounds may be obtained.

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