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ELECTROSTATIC INTERACTIONS AT CHARGED LIPID MEMBRANES. HYDROGEN BONDS IN LIPID MEMBRANE SURFACES☆

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Hydrogen-bonded structures within lipid membrane surfaces are not disrupted by water and are of thermodynamic and therefore potential structural importance in biological systems.

1. Introduction

Lipid membranes are held together by "hydrophobic bonding" between their non-polar hydrocarbon chains, and this determines their well-known bilayer structure. Such lipid bilayers undergo a reversible ordered-to-fluid transformation when heated above a "phase transition temperature", T_t. This transition, from the crystalline to the liquid-crystalline state, is accompanied by an increase in the mobility of the lipid's hydrocarbon chains and by an increase in the area occupied by each lipid molecule [1].

The transition may be regarded as a property of the hydrocarbon chains, since analogous phase transitions are shown by pure hydrocarbons (straight-chain paraffins) [2,3] and since the transition in lipid bilayers is accompanied by changes in the hydrocarbon chain lattice [4]. However the transition is influenced thermodynamically by the structural changes which it causes in the head-group region, usually associated with a change in area of the membranes. Thus effects in the head-group region at the membrane/water interface can be studied by observing changes in the phase transition temperature.

The principal property of the head-groups of lipid molecules is their polarity, resulting in their solubility in water; their interactions with water include the dielectric solvation of charged groups (such as $-NMe_3^+$ in lecithins) and the formation of hydrogen bonds between head-groups and water (as in mono- and diglycerides). Interactions such as electrostatic attraction or repulsion between polar groups in the surface of a lipid membrane do not modify the basic bilayer structure, but they can affect the behaviour of lipids in the bilayer — their conformation and thermodynamic stability, their binding of ions and molecules and their degree of crystallinity or fluidity.

An additional possible interaction between lipid head-groups is the formation of hydrogen bonds. These are known to exist between lipid head-groups in chloroform solution [5], where no bilayers are present. However, most intermolecular and many intramolecular hydrogen bonds are broken by dissolution in water. It is therefore impossible to predict whether such hydrogen bonding can still exist between the lipid head-groups of the bilayer, which in simple phospholipids are fully accessible to solvating water molecules and whose approach to one another is constrained by the bilayer structure. In addition it is difficult to demonstrate the occurrence of hydrogen bonds in the plane of the bilayer by conventional methods such as infrared and Raman spectroscopy or N.M.R., owing to the large excess of water in the experimental system. However our experiments with synthetic

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phospholipids bearing well-defined polar groups have allowed us to infer the presence of hydrogen bonds in bilayer systems. We wish in this paper not only to demonstrate hydrogen-bond interaction in phospholipid bilayer membranes, but also to show that these interactions influence the physical state of the membrane, affecting the response of the bilayer system to changes in its environment.

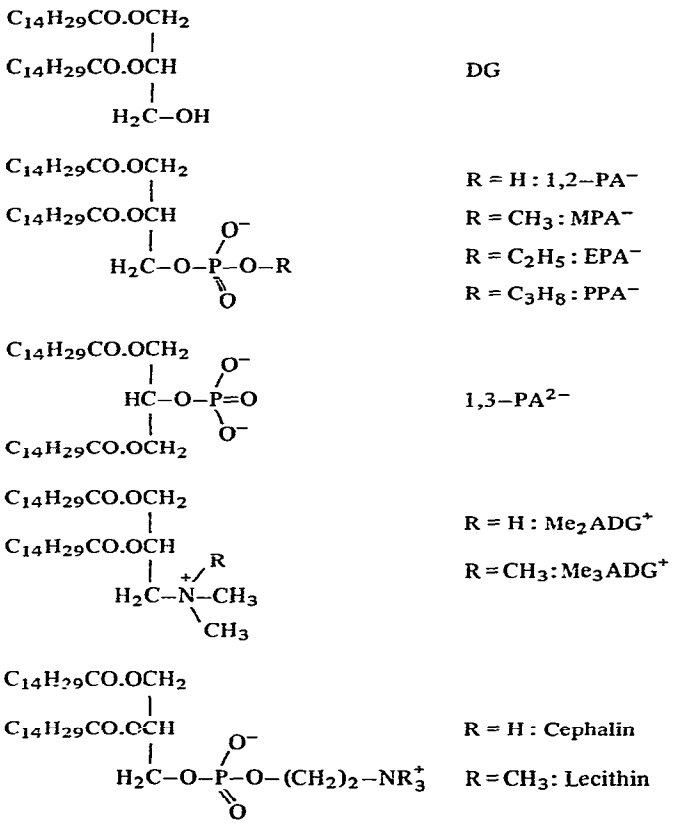


Fig. 1. The structures of some lipids. Systematic names: MPA, 1,2-dimyristoyl-methylphosphatidic acid; DG, 1,2-dimyristoylglycerine; PA, 1,2- or 1,3-dimyristoyl-phosphatidic acid; PPA, 1,2-dimyristoyl-*n*-propylphosphatidic acid; EPA, 1,2-dimyristoyl-ethylphosphatidic acid; Me_2ADG, N,N-dimethyl-2,3-dihydroxy-*n*-propylamine dimyristoyl ester; Me_3ADG^+, N,N,N-trimethyl-2,3-dihydroxy-*n*-propylammonium cation dimyristoyl ester; lecithin, dimyristoyl phosphatidyl choline; cephalin, dimyristoyl phosphatidyl ethanolamine. States of protonation are inferred from the charge, e.g. PA^{2-}(fully deprotonated), MPA^0 (protonated). The abbreviations refer to the head groups only; the hydrocarbon chains are in each case those shown here except where otherwise stated in the text.

A key to the structures of and abbreviations for the lipids referred to in this paper is given in fig. 1.

Electrostatic interaction between charged head-groups has been intensively studied [6,7]. For lipids with simple, small ionisable groups the general conclusion was: charged head-groups repel each other, destabilising the membrane in comparison with uncharged head-groups. This destabilisation, the "electrostatic free energy", was measured by means of the temperature of the crystal-liquid crystal phase transition, T_t, and a quantitative relationship was derived in which the change of T_t was expressed as a function of Δf, the change in molecular area at the phase transition [6]. The salient equation is eq. (1),

$$T_t = T_t^* + \Delta G^{el}/\Delta S^* \quad (1)$$

which for MPA at low salt concentration leads to eq. (2),

$$T_t = T_t^* - 70.5\ \alpha\ \Delta f/f \quad (2)$$

where T_t^* is the transition temperature and ΔS^* the transition entropy of the *neutral* lipid membrane; f is the area of one lipid molecule ($f \gg \Delta f$) and α is the degree of dissociation. In accordance with intuition, the mutual repulsion of the negatively charged groups favours expansion of the membrane, so that T_t is lowered by an amount proportional to the charge density ($\propto \alpha/f$) and to the expansion at the transition (Δf). Over the range $\alpha = 0$ (low pH, neutral membrane) to $\alpha = 1$ (high pH, charged membrane) we thus have for MPA a drop in T_t, given by $\Delta T_t = -70.5\ \Delta f/f$. A ΔT_t of $-18°C$ was determined experimentally for MPA, resulting in a value for $\Delta f/f$ of 0.256, which agrees well with values of 0.25 to 0.30 measured for dipalmitoyl lecithin using X-ray diffraction [1] and electron spin resonance [8].

Since the appearance of the earlier paper [6], X-ray measurements in our laboratories (kindly made available by K. Harlos and A. Blume) have shown that $\Delta f/f$ for MPA is only about 0.15, giving much worse agreement with eq. (2). The reason for this discrepancy appears to lie in hydrogen bonding, as we wish to show here.

There is much evidence that electrostatic considerations alone leave certain observations unexplained. Hydrogen bonding has been suggested as an explana-

tion [7] for the rather large difference in pK_a of phosphatidyl serine above and below its T_t, and the inadequacy of electrostatics alone has been emphasized by other authors [9].

There is much current discussion about possible hydrogen-bond interaction in the stabilisation of phospholipid–cholesterol systems [10–13] and in sphingomyelins [14], but direct demonstration of such interactions has not been possible. It is probable that the biological activity of silica [15] rests in part on hydrogen-bonding interactions involving the Si–OH group [16].

2. Experimental

2.1. Phospholipids

1,2-Dimyristoyl-sn-glycerol-3-phosphoryl-ethanolamine (cephalin) and -choline (lecithin) were products of FLUKA (Switzerland). 1,2-sn-dimyristoylglycerol and 1,3-dimyristoylglycerol were synthesized as described earlier [17]. The alkyl esters of phosphatidic acid were prepared as described in a previous paper [6]. The boiling points of the alkyl phosphoric acid dichlorides are: methyl, 44–46°C/11 mm[‡]; ethyl, 53°C/10 mm; propyl, 65°C/12 mm. The purity of the alkyl esters was checked by thin-layer chromatography and elemental analysis.

2.2. Synthesis of phosphatidic acids

Freshly distilled phosphorus oxychloride, 1.8 g (0.012 mole) was dissolved in 20 ml of trichloroethylene and cooled to 0 to 5°C. Triethylamine, 2 g (0.02 Mol), in 20 ml of trichloroethylene and then 1,2- or 1,3-dimyristoyl glycerol, 5 g (0.01 Mol), in 20 ml of trichloroethylene were added and stirring was continued.

The ice-bath was replaced by a water-bath at 20°C in the case of 1,2-sn-dimyristoylglycerol and at 40°C in the case of the 1,3 isomer. The reaction was complete after 30 minutes, as shown by thin-layer chromatography. The starting-material, with an R_f value of about 0.3 in 1 : 1 diisopropylether/hexane (v/v), was completely converted to a new substance, R_f value about 0.6, which gave a deep blue colour with molybdate spray [18]. After the addition of 60 ml of toluene the reaction mixture was filtered to remove the precipitated triethylammonium hydrochloride. The filtrate was evaporated and the residue was dissolved in 100 ml of tetrahydrofuran. Sodium hydrogen carbonate, 100 ml (0.5 *M*), and EDTA, 20 ml (0.5 *M*, pH 10.5) were added to hydrolyse the phosphorus oxychlorides. The hydrolysis was complete after 6 hours. The tetrahydrofuran layer was withdrawn and filtered and 100 ml of acetone was added to the filtrate. The precipitate was collected and repartitioned in a mixture of 450 ml of $CHCl_3$/CH_3OH/H_2O (1 : 1 : 1) to remove sodium acetate from the lipid. The chloroform layer was evaporated. The residue was dissolved in 40 ml of chloroform and 400 ml of acetone was added. The precipitate was collected. The purity of the phosphatidic acids was checked by thin-layer chromatography and elemental analysis (calculated for $C_{35}H_{67}Na_2O_8P \cdot H_2O$ (mol. wt. 710.89): C 59.14%, H 9.78%, P 4.36%; found for the 1,2-isomer: C 58.65%, H 9.85%, P 4.57%; found for the 1,3-isomer: C 58.41%, H 9.79%, P 4.43%).

2.3. Synthesis of 3-(N,N,N-trimethyl)-amino-propane(1,2)diol-dimyristoyl ester and its 3-(N,N-dimethyl)amino analogues

3-bromo-propane-(1,2)-diol, 6.2 g (0.04 mole), were dissolved in 10 ml of tetrahydrofurane and 10 ml of trichlorethylene. Triethylamine, 26 g (0.26 mole), in 10 ml of trichlorethylene was added and followed by 24.7 g (0.1 mole) of myristoyl chloride in 20 ml of trichlorethylene with stirring. The reaction mixture was filtered after the addition of 50 ml of toluene to remove triethylamine hydrochloride. The filtrate was evaporated and the residue dissolved in 100 ml of diisopropyl ether and washed with 100 ml of HCl (0.1 *N*). The diisopropyl ether phase was evaporated and the residue was purified by chromatography on 100 g Silica gel (Merck, 35–70 mesh) with solvent systems of hexane/diisopropyl ether of increasing polarity. The yield was 15 g (60% based on 3-bromo-propane(1,2)diol). The purity of the compound was checked by thin-layer chromatography and elemental analysis (calculated for $C_{31}H_{60}O_4Br$

[‡] The boiling range in Träuble et al. [6] of 44 to 46°C for the methyl ester applies for a pressure of 11 mm, and not 1 atm. as given.

(mol. wt. 576.74): C 64.56%, H 10.49%, Br 13.86%; found: C 64.30%, H 10.37%, Br 13.49%).

3-Bromo-propane-1,2-diol dimyristoyl ester, 2.4 g (0.005 mole), was dissolved in 10 ml of chloroform, 10 ml of 2-propanol and 40 ml of dimethylformamide. After the addition of 40 ml of trimethylamine (33% solution in ethanol) or dimethylamine (33% solution in ethanol) the reaction mixture was heated to 50°C for 12 hours. The solvents were evaporated and the residue was purified by chromatography. The yield of the pure product was 2.0 g (85% based on 3-bromo-propane-1,2-diol dimyristoyl ester) in the case of 3-(N,N,N-trimethyl)-amino-propane-1,2-diol dimyristoyl ester and was 1.7 g (65% based on 3-bromo-propane-1,2-diol dimyristoyl ester) in the case of 3-(N,N-dimethyl)-amine-propane-1,2-diol dimyristoyl ester. The purity of the compounds was checked by thin-layer chromatography and elemental analysis (calculated for 3-(N,N,N-trimethyl)-amino-propane-1,2-diol dimyristoyl ester, $C_{34}H_{69}NO_5$: C 71.40%, H 12.16%, N 2.45%; found: C 69.87%, H 12.01%, N 2.44%; and for 3-(N,N-dimethyl-amino-propane-1,2-diol dimyristoyl ester, $C_{33}H_{65}NO_4$: C 73.42%, H 12.14%, N 2.60%; found: C 73.11%, H 12.20%, N 2.55%).

2.4. Determination of the transition temperature

This was carried out using the fluorescent indicators NPN (N-phenyl-α-naphthylamine) and ANS (1-anilino-naphthalene-8-sulphonamide). Details are given in ref. [6]. Temperature scanning was at 1°C/minute.

2.5. Lipid mixtures

These were normally prepared by mixing lipids in the required proportion in chloroform or trichloroethylene and, after evaporation of the solvent, dispersing the residue in water or buffer. When possible, sonication was avoided.

The mixtures of MPA and DG were prepared by incubation at 70°C and pH 8. Equilibrium was reached after 2–3 days. Under these conditions some of the 1,2-DG isomerises to give the 1,3-DG isomer [19]. However, this will not affect the interpretation of the results obtained with DG (fig. 2 and below), since the number of chains is unaltered and the hydroxyl group is still present.

3. Results

3.1. MPA/DG mixtures

The behaviour of mixtures of the lipids MPA^- and DG is shown in fig. 2. T_t is plotted against the composition of the mixture. As DG is added to MPA T_t rises to 44°C. The rise stops at a molar ratio of 1 : 1 MPA : DG. The addition of further DG causes no further change in T_t, but the melting point of DG micelles is detected (ca. 35°C). As the proportion of MPA is further reduced the amplitude of the higher transition decreases (progressively fainter circles) and that of the lower transition increases. Thus fig. 2 shows on the left a continuously-variable mixture of MPA and DG, and on the right a separation into phases containing respectively approximately 50% DG and approximately 100% DG. That the separation is not quite complete is shown by the small deviation of the two right-hand branches from the horizontal.

Therefore (i) the potential proton acceptor MPA^- can incorporate up to one equivalent of the potential proton donor DG, and (ii) the mixture shows a T_t higher than that of the components, so some force must tend to reduce the membrane's area.

Further, this cannot be a mixing due to relief of steric pressure at the head-groups of either lipid, since in both cases the head-group is very small, so the area of each lipid is determined by the chain packing alone. Here the chains are identical.

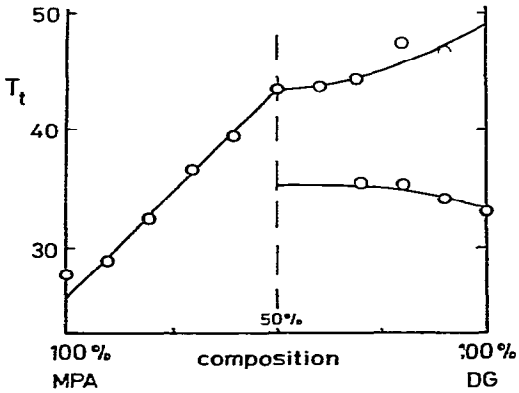
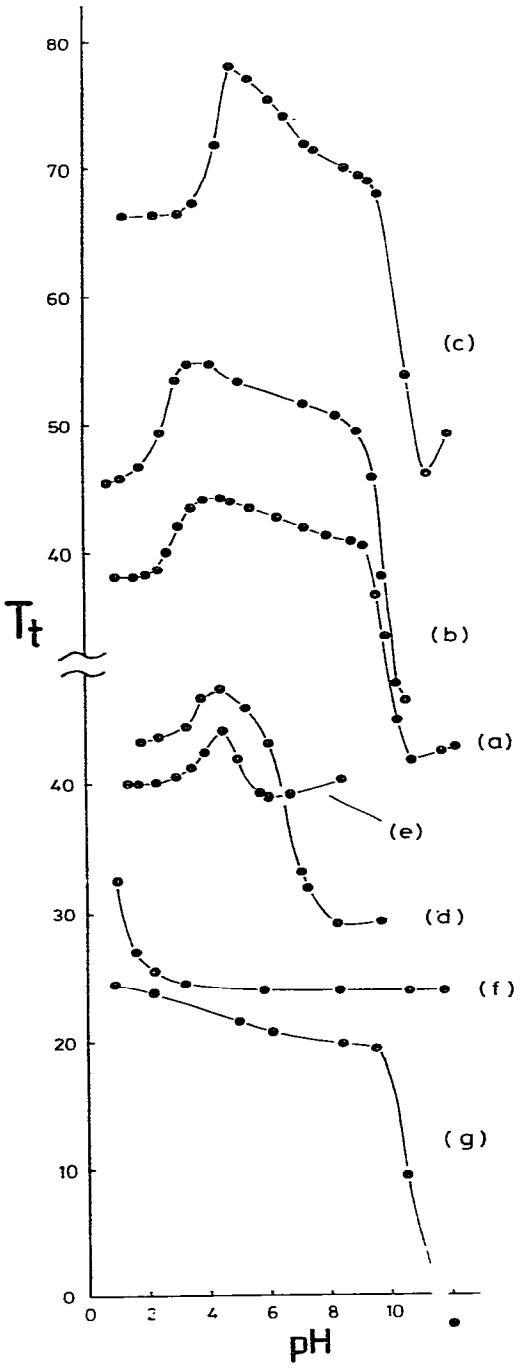


Fig. 2. Transition temperatures of mixtures of MPA^- (head-group $-OP(OCH_3)O_2^-$) and DG (head-group $-OH$).



3.2. Phosphatidic acid

The system $PA^0/PA^-/PA^{2-}$ was re-investigated. Dispersions of 1,2-PA are unstable at extremes of high and low pH, so to circumvent this problem 1,3-PA was synthesized; dispersions of 1,3-PA are chemically and physically stabler. T_t for 1,3-PA^0 is 38°C and for 1,3-PA^{2-} 24°C (fig. 3a); the drop of 14°C is due to electrostatic repulsion between the headgroups and corresponds (eq. (1)) to a relative change, $\Delta f/f$, in the molecular area of 10%. However, the change is not smooth; partially protonated states show much higher T_t values. Within the measurable range curves of identical form were obtained both for 1,2-PA (fig. 3b), lying a few degrees higher on account of more efficient packing in the hydrocarbon layer, and for the analogue of 1,2-PA (fig. 3c) in which the long-chain esters are replaced by the much stabler long-chain ethers, making the measurements proof against artefacts due to lipid decomposition. As in fig. 2, the high T_t value of partly-protonated membranes can be attributed to a structure-stabilising P–OH...$^-$O–P bond.

3.3. Alkyl phosphatidic acids

Previous investigations of alkyl phosphatidic acids had failed to reveal such behaviour [6,10,11], but it can indeed be found, as figs. 3d and 3e show for the cases of MPA and PPA. The left-hand branch of the curve had not previously been seen because at middling and high salt concentrations ($\geqslant 10^{-3}$ molar) the salt-induced shift of the titration curve of MPA pushes this branch into the region where lowering the pH also raises the salt concentration, making further measurements impossible. EPA behaves similarly to MPA. a few degrees lower; PPA^0 shows the same T_t value as PPA^-, on account of steric effects not fully understood [21], but the stabilisation in the partly protonated state is clear (fig. 3e). Here the addition of salt again pushes the entire curve over to the left, making the left branch unmeasurable.

The melting-point of pure microcrystalline, fully-

Fig. 3. Variation of transition temperature with pH for different lipids (a) 1,3-PA; (b) 1,2-PA; (c) 1,2-PA (dipalmitoyl diether analogue); (d) MPA; (e) PPA; (f) lecithin; (g) cephalin. Measurements were made at low ionic strength (< 1 mM), using aqueous dispersions typically 10^{-4} M in lipid.

protonated MPA recrystallised from nonaqueous solvents also lies in the range 40–50°C, in contrast to that of the crystalline sodium salt of MPA, which is 260°C (H. Eibl, unpublished observations). The transition which we observed at 42°C was reversible and the addition of salt raised it to 47°C, as expected, so we ascribe it to the crystalline-liquid crystalline transformation and not to a complete melting of the protonated bilayers. Even if this were incorrect, the conclusion that ascent of the right-hand branch of the T_t/pH curve in fig. 3d does not correspond to full protonation (see "Discussion") remains unaffected.

3.4. Other phospholipid systems

Table 1 shows a set of further transition temperatures measured. In addition, the pH-dependence of T_t for dimyristoyl lecithin and 1-pamitoyl-2-oleoyl cephalin is shown in figs. 3f and 3g. These observations are discussed below.

Table 1

Lipid	Condition	T_t °C
Me_2ADG	pH 2–4	39
Me_2ADG	pH 7–9	12
Me_3ADG^+/MPA (1 : 1)	neutral pH	29
Me_2ADG^+/MPA (1 : 1)	neutral pH	50

4. Discussion

4.1. The behaviour of MPA/DG mixtures

In fig. 2 it is seen that the potential proton acceptor MPA^- can incorporate up to one equivalent of the potential proton donor DG, stabilising the packing in the membrane structure (higher T_t). The only likely origin of such a specific, one-to-one stabilisation of the structure is the formation of hydrogen bonds between the hydroxyl and phosphate groups.

We therefore attempted a systematic examination of systems in which hydrogen bonds between lipids might be found. We use the term *interfacial* to denote hydrogen bonds spanning the lipid-water interface, i.e., bonds between lipid and water, and *intrafacial* to denote hydrogen bonds within the lipid surface, i.e., between adjacent lipid head-groups.

The reason for the apparent discrepancy between the T_t behaviour of MPA and the electrostatic theory (above) is now clear. The attribution of ascent of the right-hand branch of fig. 3d to a complete protonation of the lipid was incorrect; protonation is only complete on the left of the left-hand branch. Thus T_t for MPA^0 is not 47°C, but only 42°C, resulting in a $\Delta f/f$ value for MPA of 0.18, now in much better agreement with that of 0.15 obtained from X-ray measurements with MPA.

The biphasic T_t/pH curve also suggests that the quantitative titration of MPA^- with acid in the presence of salt will not reveal the left-hand branch, so that the number of protons taken up by an MPA^- molecule will appear to be one-half. This is borne out by an earlier result of M. Teubner (ref. [22] and personal communication), who carried out the titration in 10 mM NaCl and obtained precisely this result.

4.2. Hydrogen bonds in other systems

Since it now seems clear that intrafacial hydrogen bonds can make an important contribution to the free energy of a membrane surface, we review some systems in which unexpected behaviour may be explained by assuming that intrafacial hydrogen bonds exist.

1) Amine-lipids with the polar head-group $-N(CH_3)_2$ may be protonated by acid, thus acquiring a positive charge, which according to the electrostatic theory should depress the T_t value. However the lipid Me_2ADG has a T_t value of 39°C (table 1) in the pH range 2.0–4.0 and only 12°C in the pH range 7.0–9.0, which is the reverse of the behaviour expected. We propose that when the neutral membrane starts to take up protons, intrafacial $\geq\overset{+}{N}H\ldots N\leq$ links form whose stabilisation of the structure exceeds the destabilisation caused by the build-up of positive charge. Full protonation of the membrane is not achieved under the conditions of the experiment since this would require the breaking of the strong intrafacial links and a still greater build-up of positive charge; this system will be the subject of further investigation to determine the magnitude of the effects concerned.

2) The lipids Me_3ADG^+ and MPA^- are singly charged at pH 5 in 0.1 M NaCl and possess corre-

sponding T_t values, viz., around 25°C for dimyristoyl chains. Me_2ADG^+ is partly charged. When MPA^- and Me_3ADG^+ are mixed in equal proportions, the mixed membrane is neutral and a T_t of 29°C is seen (table 1). However a similar mixture of MPA^- and Me_2ADG^+ has a far higher T_t, 50°C. This means not only that the structure-expanding electrostatic influence is absent in the neutral mixture, but that also that the compact, crystalline state is further stabilized when H-bond formation is possible, i.e., in $R_3\overset{+}{N}$–H...$^-O_4PR_2$ but not between $R_4\overset{+}{N}$ and $^-O_4PR_2$.

3) It is not possible within the normal pH range to protonate the phosphate group of lecithin in order to observe the effect of charging up the membrane [23]. However the onset of protonation can be observed, and the T_t value *rises* accompanying proton binding (fig. 3f), although the electrostatic theory predicts that it should fall. This rise can be explained by postulating PO_4^-...H–O_4P bridges, whose stabilising effect at first exceeds the electrostatic destabilisation of the structure, just as in the initial deprotonation of the phosphatidic acids and in the initial protonation of Me_2ADG.

4) The fact that T_t for dimyristoyl lecithin is 23°C and T_t for dimyristoyl cephalin is 50°C has often been attributed to the reason that the bulky lecithin dipoles are sterically constrained to lie perpendicular to the membrane surface, bringing like charges together and thereby repelling one another, while the smaller cephalin dipoles can lie slanted, bringing opposite charges together and attracting one another. This view has been refuted by Seelig [24] whose isotope studies have shown that the lecithin dipoles are also slanted. The only remaining additional stabilisation available to cephalin lies in the hydrogen bonds between the $-\overset{+}{N}H_3$ and PO_4^- groups, and indeed the surest test of hydrogen bonding, viz., bond-shortening observed by X-ray crystallography, has been carried out in this case [25]; intrafacial, intermolecular hydrogen bonds were seen in bilayers dilauroyl cephalin crystallised from glacial acetic acid.

To observe the pH-dependence of T_t shown by cephalin 1-palmitoyl-2-oleoylcephalin was used, on account of the high stability of its bilayers at low pH. The result is shown in fig. 3g. The decrease of ca. 25°C seen upon deprotonation results from the combined effects of greatly weakening the hydrogen bonds and charging up the surface. The effect of initial protonation is more complex; the amino-phosphate hydrogen bonds are broken while phosphate–phosphate hydrogen bonds are formed and the phosphate groups are neutralised. The resultant of these is a marginal increase in T_t from 22°C at pH 7 to 24°C at pH 0.5. Complete protonation is not achieved.

4.3. Bond-weakening at the phase transition

The starting point for a theoretical consideration of the influence of intrafacial hydrogen bonds upon the phase transition of a lipid is the Gibbs' molar free energy of the membrane, given approximately by $G = G^* + G^{el} + G^h$, where G^* refers to the uncharged state with no stabilising hydrogen bonds and G^{el} and G^h are respectively the contributions of the electrostatics and of hydrogen bonding. G^{el} is necessarily positive and G^h negative. Using the method of ref. [3], we obtain eq. (3), analogous to eq. (1).

$$T = T_t^* + \Delta G^{el}/\Delta S^* + \Delta G^h/\Delta S^* \qquad (3)$$

Since the term $\Delta G^h/\Delta S^h$ is of the order of 10°C (fig. 3), and ΔS^* is typically 20 cal./mole/deg. (83.7 J./mole/deg.), ΔG^h must be small, ca. 200 cal./mole (837 J./mole), although G^h could be much greater.

If intrafacial hydrogen bonds raise the transition temperature, this means that the crystalline state is more stabilised by these than is the liquid-crystalline state. We may therefore picture two extreme cases: (i) there are a few, weak hydrogen bonds, which at the transition to liquid-crystalline are broken; (ii) there are many, strong hydrogen bonds, which at the transition become weaker. (Here "weak" means in fact "stronger than the competing interfacial bonds by an energy $\sim kT$" and "strong" means "stronger than these by an energy $\gg kT$"). Either effect would be due simply to stretching of the bonds accompanying expansion of the lipid membrane. Note that the very low value of ΔG^h rules out the possibility of strong hydrogen bonds of which all are broken at the transition.

Now weak hydrogen bonds would only involve a few of the available protons, so the protonation constant would hardly be changed and the number of intrafacial hydrogen bonds would be proportional‡ to $\alpha(1-\alpha)$, with a constant of proportionality changing

‡ See page 268.

at the transition. Strong bonds would involve all available protons and proton acceptors, so that hydrogen bonding would increase the protonation constant when $\alpha > 1/2$ and decrease it when $\alpha < 1/2$, while the number of hydrogen bonds would be proportional to α or $1 - \alpha$, whichever were the smaller, and would not change at the transition (only their strength would change). Experimental lines of evidence indicating that the strong-bond picture is nearer to the truth are (1) the linearity of the rise in T_t in the left-hand part of fig. 2, (2) the approximate invariance of the magnitude of the stabilisation when several different head-groups are compared (fig. 3), and (3) the apparent splitting of the titration curve of MPA and PPA into branches separated by two pK units or more (figs. 3d and 3e, and the observation of M. Teubner mentioned above).

We now make an order-of-magnitude calculation to investigate whether the sort of effects postulated could be responsible for increasing T_t around ten degrees. A half-protonated lipid membrane is considered, and we assume: (i) that the breaking of an intrafacial hydrogen bond and the solvation by interfacial hydrogen bonds of the groups thus freed is accompanied by an energy term u_h; this must be positive, as a negative value would stop intrafacial hydrogen bonds from being formed at all; (ii) that interfacial hydrogen bonds are statistically preferred, owing to the freedom of movement and high concentration of the water molecules, by a factor ξ; (iii) that P possible hydrogen-bonding pairs exist, of which n form hydrogen bonds. Then using Boltzmann statistics, we obtain eq. (4).

$$n = \frac{P}{1 + \xi \exp(-u_h/kT)} = \frac{P}{1 + \zeta} \tag{4}$$

ζ is thus the "disruption constant" of an intrafacial hydrogen bond. If free energy per hydrogen bond is given by g_h, then for one mole of half-protonated lipid ($P \approx L/2$, L is Avogadro's number), we have eq. (5).

$$G^h = n g_h = \frac{1}{1 + \zeta} \cdot \frac{L}{2} \cdot g_h \tag{5}$$

Differentiating, we obtain at the transition equation (6).

$$\frac{\Delta G^h}{\Delta f} \approx \frac{dG^h}{df} = n \frac{\partial g_h}{\partial f} + g_h \frac{\partial n}{\partial f} \tag{6}$$

The first term represents bond-weakening and the second bond-breaking. A detailed calculation, given in the appendix to this paper, shows that the second term is zero, with enthalpic and entropic components cancelling one another out, while the first term becomes $(-n\, du_h/df)$. Thus even in the weak-bonding case (above) the raising of T_t is due to bond-weakening and not to bond-breaking, even though bonds are broken at the transition.

To obtain du_h/df we require a physical model of the individual hydrogen bonds, and shall assume that the proton donor is a dipole of moment M situated at a distance $\sqrt{f/2}$ from and directed towards the proton acceptor, which is a single elementary negative charge e. The energy of interaction is $-u_h = -2Me/\epsilon f$ (ϵ is the dielectric constant). A constant term should of course have been added to include the competition from water, but this will disappear again in the derivative du_h/df. The third term in eq. (3), which gives the direct effect upon T_t of the hydrogen bonding, is then obtained by integrating eq. (6). The exact result is cumbersome, and eq. (7) is a good approximation for all values of ζ.

$$\frac{\Delta G^h}{\Delta S^*} = \Delta T_t^h = \frac{L \Delta f}{2 \Delta S^*} \cdot \frac{1}{1 + \zeta} \cdot \frac{Me}{\epsilon f^2} \tag{7}$$

Inserting values of $M = 1.7 \times 10^{-18}$ e.s.u. (typical O–H bond moment), $e = 4.8 \times 10^{-10}$ e.s.u., $\epsilon = 3$ (since there is no intervening water), $f = 40$ Å^2, $\Delta f = 6$ Å^2 and $\Delta S^* = 17.5$ cal/mol/deg (the last three values applying to MPA) yields for low ζ values $\Delta T_t^h \approx 8$°C, which in view of the fact that no parameters were fitted is satisfactory (with such a crude model any result between 1°C and 100°C would have been acceptable).

4.4. Conclusion for biological membranes

While it is clear that the polar head-groups of a lipid membrane undergo hydrogen bonding, it is not

‡ This assumes that the distribution of protonated and unprotonated lipids is random, which is indeed to be expected if the hydrogen bonds are weak, i.e., they cannot impose an ordered structure onto the head-groups. A non-random structure could also result in separation into two phases, which however was not observed in the studies summarised in fig. 3.

clear a priori whether intrafacial bonding between polar groups can occur or whether competition from water molecules rules this out. Our experiments have shown that intrafacial hydrogen bonds can indeed make a significant contribution to the energy of the membrane when the lipids possess a suitable proton donor ($\dot{N}H$, OH) and proton acceptor (N: , PO_4^-).

Such bonding would be a factor to be taken into consideration in situations where interactions between head-groups play a part in determining the behaviour of a biological or an artificial lipid membrane, such as those involving diffusion or dissociation of single molecules, crystalline-liquid crystalline transitions, phase separation or domain formation. Biological membranes possess, in addition to the heterogeneity of their lipids, the further complicating features that sugars and proteins with structural or catalytic functions are also built into these membranes, and that smaller molecules may also be transported within the membrane phase; intrafacial hydrogen bonding could make an important contribution to the uptake mechanism and binding strength of such guest molecules. Hydrogen bonds between molecules in aqueous systems often receive little emphasis, partly because they are assumed to be unimportant in comparison with hydrogen bonds to water and partly because they are extremely difficult to detect. Our studies using the lipid phase transition as a principal (though indirect) means of detection have shown that hydrogen-bonded structures at lipid membrane surfaces are not disrupted by water and are of thermodynamic — and therefore potentially of structural — importance, so that, in spite of the present difficulty in characterising them, their rôle as mortar between the bricks of biological walls should not be ignored.

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Appendix

Why bond-weakening and not bond-breaking at the phase transition determines the influence of intrafacial hydrogen bonds upon the transition temperature.

We picture the proton donor as a vector free to point in any direction permitted by the covalent bonds of the lipid molecule of which it is part. If no proton acceptor is nearby, all permitted directions result in states of equal energy, whether or not they are hydrogen-bonded to (structureless) water in the aqueous phase. A proton acceptor is now allowed to approach the proton donor and to reside at a distance from it suitable for the formation of a hydrogen bond. This means that pointing in some directions — approximately towards the proton acceptor — will give the system a lower internal energy than if the proton acceptor were not there, while pointing in any other direction will not change or may even raise the internal energy of the system. We now consider the change in free energy which the system undergoes when the acceptor approaches, bearing in mind that in general the hydrogen bonds may be weak, in which case a distribution of vector directions can be expected. The simplest possible case will be treated, and its extension to more realistic situations described afterwards.

We suppose that the approach of the proton acceptor results in the permitted directions of the proton donor being divided into two classes, those with the same internal energy as before and those with an internal energy lower by an amount u. The ratio of the solid angles containing these directions is ξ, and this corresponds exactly to the ξ introduced in the text above and in eq. (4).

Before the approach of the proton acceptor the molecular free energy of each of the P proton donors is given by g. If g^0 is the standard molecular free energy of the proton donors when pointing in a fixed direction then eq. (A1) results.

$$g = g^0 + kT \ln \frac{aP}{\xi + 1} \qquad \text{(A1)}$$

In this expression the second term corresponds to the entropic term encountered in dilution, and a is a constant introduced to correct the dimensions and the arbitrary choice of the standard state; the value of a is unimportant.

After the approach of the proton acceptor, out of a possible total of P hydrogen bonds, n are formed. The molecular free energy of the free vectors must therefore be rewritten as in eq. (A2) and that of the

bound vectors is given in eq. (A3).

$$g_f = g_f^0 + kT \ln \frac{a(p-n)}{\xi} \quad \text{(A2)}$$

$$g_b = g_b^0 + kT \ln \frac{an}{1} \quad \text{(A3)}$$

However, the internal energies have changed by the amounts u and zero respectively, so $g_f^0 = g^0$ and $g_b^0 = g^0 - u$. Therefore for the whole system the change in free energy upon approach of the proton acceptor to the proton donor is given by the right-hand side of eq. (A4), and this is identical to the free energy of hydrogen bond formation, G^h, introduced in the text and given by eq. (5).

$$G^h = -Pg + (P-n)g_f + ng_b \quad \text{(A4)}$$

Inserting the values for g_f, g_b and g from eqs. (A1)–(A3) into eq. (A4) leads to eq. (A5) for G^h. It will be noted that if both sides of eq. (A5) are differentiated with respect to n and dG^h/dn is set equal to zero then the equilibrium condition is derived (eq. (4)).

$$G^h = -nu - PkT \ln \frac{P}{\xi + 1} + (P-n)kT \ln \frac{P-n}{\xi} + nkT \ln n \quad \text{(A5)}$$

At the phase transition the molecular area f of the lipid changes. This affects the value of G^h in eq. (A5), and the change ΔG^h results in a contribution to the change in transition temperature from the hydrogen bonds, as expressed in eq. (3) of the text. Writing as an approximation for small changes in the molecular area $\Delta G^h = (dG^h/df)\Delta f$, it follows that the effect of the intrafacial hydrogen bonds on the transition temperature is directly proportional to dG^h/df, as given in eq. (A6).

$$dG^h/df = -n\, du/df - u\, dn/df + \frac{d}{df}\left(-PkT \ln \frac{P}{\xi+1} + (P-n)kT \ln \frac{P-n}{\xi} + nkT \ln n\right). \quad \text{(A6)}$$

After manipulation this reduces to eq. (A7). Comparing the term in brackets with eq. (4) it is seen that this term is simply equal to u, resulting in eq. (A8).

$$\frac{dG^h}{df} = -n \frac{du}{df} - u \frac{dn}{df} + \frac{dn}{df} kT \left(\ln \frac{n\xi}{P-n}\right) \quad \text{(A7)}$$

$$dG^h/df = -n\, du/df \quad \text{(A8)}$$

This unexpectedly simple relationship shows, as stated above, that the raising of T_t by hydrogen bonds is due to bond-weakening and not to bond-breaking, even if bonds are broken at the transition.

This simple calculation does in fact cover more complex systems. Firstly, it is not necessary to assume as we did that the approach of the proton acceptor produces only two energy levels for the proton donor. In fact a virtually continuous distribution of levels will appear. These may be included in the calculation by considering an arbitrary number of states, all of which are degenerate in the absence of the proton acceptor and which have internal energy levels u_0, u_1, u_2 etc. and degeneracies ξ_0, ξ_1, ξ_2 etc. in its presence. For each additional state an extra equation such as (A2) or (A3) is written, a term is added to eq. (A4) and, following the same procedure, the same equation (A8) is reached. Secondly, it is not necessary to assume that the proton donor group and the proton acceptor group remain at a fixed distance from each other, irrespective of the orientation of the proton donor vector; variations in distance apart simply result in further energy levels, and these may be taken account of in the same way.

Apart from the insight which it provides into the mechanism of the stabilisation of the ordered state of the lipid by intrafacial hydrogen bonds, this result is of interest in that it confirms the general prediction made in a previous paper [6] that any property of the membrane surface remaining in an equilibrium state during the phase transition – even if the transition moves the point of equilibrium – makes no contribution to the transition temperature as long as the intrinsic equilibrium constant for the binding of protons to the phosphate groups remains unchanged. In the earlier work this conclusion was applied only to the degree of dissociation, but there is no reason to restrict it to this. Here the *breaking* of hydrogen bonds reflects the shift in the equilibrium between broken and intact intrafacial hydrogen bonds at the transition. This, for the reason stated, does not affect the transition temperature. However the *weakening* of the intrafacial hydrogen bonds changes the intrinsic protonation constant of the head-group, so this weakening can affect T_t..

Note added in proof: The use of fluorescent indicators has been criticised by a referee on the grounds that they may cause artefacts due to their incorporation into the lipid. Since the completion of this work a detailed study of the equilibria and kinetics of NPN binding to MPA has been carried out (P. Woolley and H. Dieber [26,27]) and many of the T_t measurements reported here have been checked using light-scattering (H. Eibl, A. Niksch, K. Harlos, ms. in preparation). No grounds have been found for the belief that NPN causes artefactual transitions or leads to more than minor errors in T_t. The behaviour of many of the lipids in fig. 3 has been checked. Agreement is usually exact and the profiles are always qualitatively reproduced.

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