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Gregor Cevc. Regulation of the colloidal and phase behaviour of bioaggregates by surface polarity. Examples with lipid bilayer membranes. *Journal de Physique*, 1989, 50 (9), pp.1117-1134. <10.1051/jphys:019890050090111700>. <jpa-00210981>

**HAL Id: jpa-00210981**

**<https://hal.science/jpa-00210981v1>**

Submitted on 4 Feb 2008

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HAL Authorization

Classification

Physics Abstracts

87.10 — 87.15 — 87.20

## Regulation of the colloidal and phase behaviour of bioaggregates by surface polarity. Examples with lipid bilayer membranes

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(Reçu le 16 août 1988, révisé le 4 janvier 1989, accepté le 9 janvier 1989)

**Résumé.** — On dispose maintenant de beaucoup de données sur les propriétés de biomacromolécules, mais la compréhension des principes physiques qui les expliquent reste fragmentaire. En particulier, le groupe de tête, le pH et la dépendance du sel des transitions de phase lipide et de la fusion ont provoqué de nombreuses explications qui ne sont pourtant que partiellement correctes. Les résultats expérimentaux présentés dans ce travail suggèrent une interprétation simple et générale : l'explication essentielle du comportement de la phase et de la capacité de fusion de bicouches de lipides est liée à l'hydratation effective des groupes de têtes des lipides. Ceci est avant tout une fonction de la polarité effective en surface et, essentiellement, d'origine quantique. Les effets directs dus à la charge des lipides et des liaisons hydrogène entre molécules de lipides sont également présents, mais sont petits. On propose une théorie simple de la description des propriétés thermodynamiques et colloïdales des macromolécules et supramolécules solvatées. Ce modèle utilise, comme paramètres, l'hydratation interfaciale effective et les potentiels électrostatiques, ainsi que la capacité des molécules à former des liaisons entre elles. En le combinant avec une théorie de perturbation du comportement de la phase, ce modèle décrit de manière presque quantitative les propriétés de la phase et de la fusion des bicouches pour tous les glycérophospholipides courants en fonction du groupe de tête, du pH, de la concentration en sel et de l'hydratation. En outre, à cause de son caractère général, la théorie proposée fournit aussi une base pour la description des propriétés colloïdales et physicochimiques d'autres systèmes biomacromoléculaires.

**Abstract.** — Data concerning the colligative and phase properties of various biomacromolecules are now quite extensive but understanding of the physical principles that govern them is still fragmentary. In particular the head-group, pH-, and salt-dependence of lipid phase transitions and fusion have, because of their potential biological implications, provoked numerous, albeit so far only partly adequate explanations. Experimental results presented in this work suggest a simple and general interpretation : the main determinant of the phase behaviour and fusing ability of lipid bilayers is the effective hydration of the lipid headgroups. This is primarily a function of the effective surface polarity and predominantly of quantum-mechanical origin. Direct effects of the net lipid charge and of hydrogen bonding between the lipid molecules are present but are smaller. A simple unified theory for the description of the thermodynamic and colloidal properties of solvated macro- and supramacromolecules is proposed. This model uses the effective interfacial hydration and electrostatic potentials as well as the ability of molecules to form mutual bonds as parameters. In combination with a perturbation theory of the phase

behaviour this model accounts nearly quantitatively for the headgroup, pH-, salt-, and hydration dependence of the phase and fusion properties of bilayers, for all common glycerophospholipids. Moreover, owing to its general character, the proposed theory provides a basis for the description of the colloidal and physicochemical properties of other biomacromolecular systems as well.

## 1. Introduction.

Many intermolecular and macromolecular reactions, and essentially all processes in living systems occur in the presence of solvents, usually water. Supramolecular aggregates, such as membranes, can act as reaction matrices, the properties of which may depend on solvent effects. For example, amphiphilic molecules, such as lipids, spontaneously aggregate, often into bilayers, in aqueous solutions due to the tendency of the hydrophobic regions to avoid water. It is thus largely the hydrocarbon *chains* which renders the supramolecular lipid bilayer structure stable, whereas the interaggregate interactions and the colloidal stability of lipid vesicles are largely a function of the nature of the polar *headgroups and of their bathing solution*.

To date only the former, the principle of regulation of the lipid bilayer properties by the chains, is well understood [1, 2]; the molecular mechanisms by which the lipid headgroups influence the membrane characteristics are still an enigma. In this contribution I therefore try to give an answer to the latter question. I show how this and similar problems can be treated within the framework of a phenomenological molecular-force model which combines recently developed models of solvation and of generalized van der Waals forces with a standard description of surface electrostatics. I provide evidence for the interdependence of the colloidal and the phase behaviour of solvated biomolecular systems and identify the most important physicochemical factors by which these properties are controlled for lipid bilayers. Finally, I present experimental and theoretical evidence supporting the view that the solvation of noncharged (biomacro)molecular systems is largely of quantum-mechanical origin.

## 2. Energetics and phase behaviour of lipid bilayers.

**2.1 HYDROCARBON CHAIN REGION OF THE MEMBRANE.** — The major part of the bilayer free energy stems from the lipid chains. This contribution increases with the length of the hydrophobic part of the molecule,  $n_c$ , so that the temperature at which a lipid membrane changes its phase state, is on the absolute temperature scale determined chiefly by the chains [3].

The chain-melting phase transition temperature of diverse lipids falls typically in the range 250-400 K [4, 5]. For the similar chainlength homologues of various non-hydrated phospholipids the measured values differ only little; for the lipids with 12 to 22 carbons per chain they lie between 340 and 375 K. Specifically for the anhydrides of glycerophospholipids with eighteen carbons per chain the chain-melting phase transition temperature is  $T_{m, \text{anh}} \approx 370 \pm 5$  K [8]. (The full chainlength dependence of this transition temperature is phenomenologically described by the approximate relation:  $T_{m, \text{anh}}(n_c) \approx [1 - (n_c - 5.5)^{-1}]$  400 K). It is noteworthy that hydrocarbon unsaturation lowers the chain-melting transition temperature substantially, relative to the corresponding fully saturated lipids, because it causes the chain packing to be looser.

**2.2 POLAR REGION OF THE MEMBRANE.** — In contrast to the lipid anhydrides, the chain-melting phase transition temperatures of various hydrated lipids cover a much wider range of

values between  $< 290$  and  $330$  K or between  $305$  and  $350$  K for saturated chains with fourteen and eighteen carbon atoms per chain, respectively [8]. Such a spread, albeit small on the absolute temperature scale, is significant in biological systems.

From the point of view of energetics this reflects the fact that the bilayer free energy consists of two contributions, one stemming from the chains and the other from the polar region of the membrane :  $G_b = G_{ch} + G_p$ . Owing to the modifications in the intermolecular interactions and to the changes in the molecular dimensions and shape of the lipid at the phase transition this free energy changes by an amount  $\Delta G_{ch} + \Delta G_p$ . This affects the phase behaviour of the system. One of the effects of the polar membrane part is then to shift the transition temperature of a hydrated bilayer, relative to the transition temperature of a reference state. If the latter is taken to be only chain dependent, the corresponding phase transition temperature shift,  $\Delta T_t = T_{t,ref} - T_t$ , is determined mainly by the change  $\Delta G_p$ .

Specifically, for a first order phase transition, such as lipid chain-melting, the total bilayer free energy change is zero. In such case the transition temperature shift can be derived from perturbation theory (cf. Refs. [6, 8]) to be

$$\Delta T_{m,p} = \Delta G_{p,m} / \Delta S_{anh,m} \quad (1)$$

The reference transition entropy change is identified with the value typical of the lipid anhydride,  $\Delta S_{ref,m} = \Delta S_{anh,m}$ .<sup>(1)</sup> Such choice of the reference state is justified by the insensitivity of the chain-melting phase transition temperature of the lipid anhydride to the headgroup effects. The required value for the entropy change can be calculated from the phenomenological expression :  $\Delta S_{anh,m} \approx 2(n_c - 5.5)(7.5 \pm 1.5) \text{ J mol}^{-1} \text{ K}^{-1}$ , within the framework of the approximation used previously to describe the chainlength dependence of the chain-melting phase transition temperature of the dry phospholipids. For phospholipids with eighteen carbon atoms per chain the value of this parameter is approximately  $\sim 185 \text{ J mol}^{-1} \text{ K}^{-1}$ . Equation (1) is exact if for the lipid anhydrides  $G_p = \Delta G_p = 0$ .

The total change of the free energy of the polar membrane part upon chain melting is typically negative,  $\Delta G_{p,m} < 0$ . So must then also be the value of the shift  $\Delta T_{m,p}$ . Hydrated lipid bilayers consequently melt at lower temperatures than the corresponding lipid anhydrides [8-10]. The difference decreases with the hydrocarbon chainlength [8] and increases with the headgroup polarity, owing to the greater magnitudes of  $\Delta S_{anh,m}$  and of  $\Delta G_{p,m}$ , respectively (see Fig. 1 and further discussion).

### 3. The sources of the shifts in the chain-melting phase transition temperature.

In general, lipid molecules interact with each other, with ions and with the solvent, which is typically water. The bilayer free energy of the polar part of the membrane, which contains contributions from all these interactions, can then be written formally as

$$G_p = G_{bond} + G_{el} + G_h + G_{vdW} + \dots \quad (2)$$

where  $G_{bond}$  arises from the interlipid bonds,  $G_{el}$  from the electrostatic ion-lipid and certain

<sup>(1)</sup> The accuracy of the theoretical description of the bilayer phase behaviour in *excess solution* is essentially the same for each choice of the reference state, because fully hydrated phospholipids have similar values of the transition enthalpy and entropy. When comparing the phase behaviour of lipids in excess solution, any chain-melting transition temperature may therefore be chosen to represent the reference state ; to describe the effect of water concentration on  $T_m$  it must conversely be taken that  $T_{m,ref} = T_{m,anh}$ .

ion-ion interactions,  $G_h$  from the lipid headgroup hydration,  $G_{vdW}$  from the generalized van der Waals forces, etc. Based on equations (1, 2) the shift of the bilayer chain-melting phase transition temperature,  $\Delta T_{m,p}$ , also may be taken to be a sum of several contributions [7], each corresponding to one of the above free energy terms

$$\Delta T_{m,p} = \Delta T_{m,bond} + \Delta T_{m,el} + (\Delta T_{m,h}^{PO_4} + \Delta T_{m,h}^H + \Delta T_{m,h}^{=O}) + \Delta T_{m,vdW} + \dots \quad (3)$$

except that, for the sake of clarity, the hydration shift is here subdivided into three parts (in brackets). The first one is associated with the phosphate (de) protonation,  $\Delta T_{m,h}^{PO_4}$ ; the second reflects the thermodynamic consequences of a change in lipid hydration, arising from changes in the number of available protons on a (charged) headgroup by one,  $\Delta T_{m,h}^H$ , and the third,  $\Delta T_{m,h}^{=O}$ , pertains to the carbonyl-group hydration.

By means of equation (3) the differences in the transition temperatures of various lipids in excess solution can then be analyzed and used predictively [7]. Shift values can be assigned on the basis of experimental data (Tab. I). For example for the glycerophospholipids with eighteen carbon atoms per chain one then gets:  $(\Delta T_{m,bond} \sim 1 \pm 0.75 \text{ K}) \sim$

Table I. — *Chain-melting phase transition temperature (°C) <sup>(1)</sup> of bilayers of various 1,2-distearoyl-sn-glycerophospholipids <sup>(2)</sup> as a function of the head-group structure, degree of methylation, or protonation as regulators of the interfacial polarity and hydrophilicity.*

Lipid <sup>(2)</sup>	pH ≤ 1	pH = 7	pH = 13	Lipid	pH ≤ 1	pH = 7	pH = 13
DSPA	69 <sup>(3)</sup>	66	59				
DSPA(CH <sub>2</sub> ) <sub>2</sub> H	68 <sup>(3)</sup>	57.5	57.5				
DSPA(CH <sub>2</sub> ) <sub>2</sub> H	67.5 <sup>(3)</sup>	57	57	DSPA(CH <sub>2</sub> )(COH)H	67.5	56	56
DSPA(CH <sub>2</sub> ) <sub>3</sub> H	67 <sup>(3)</sup>	56.5	56.5	DSPA(CH <sub>2</sub> )(COH) <sub>2</sub> H	66	55	55
DSPA(CH <sub>2</sub> ) <sub>4</sub> H	66.5 <sup>(3)</sup>	55	55	DSPA(CH <sub>2</sub> )(COH) <sub>3</sub> H	65	54	54
DSPS	76	68	48	DSPS(CH <sub>3</sub> )	71	68	53
DSPE ≡				DSPE(CH <sub>3</sub> )	72	69	56.5
DSPA(CH <sub>2</sub> ) <sub>2</sub> NH <sub>3</sub>	76	74	56	DSP(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>3</sub> ) <sub>3</sub>	64 <sup>(4)</sup>	55	55
DSPA(CH <sub>2</sub> ) <sub>4</sub> NH <sub>3</sub>	70	63	55.5	DSPA(CH <sub>2</sub> ) <sub>4</sub> N(CH <sub>3</sub> ) <sub>3</sub>	63 <sup>(4)</sup>	54	54

<sup>(1)</sup> Phase transition temperatures were determined calorimetrically or by measuring the optical density at 300-400 nm of lipid solutions (pH = 7, > 12) or samples contained in flat mica containers (pH = 7, < 1). The values given were obtained from the heating scans, performed at a scanning-rate of ≤ 1 K min<sup>-1</sup>, and are rounded to within 0.5 K.

<sup>(2)</sup> DS ≡ 1,2-distearoyl-*sn*-glycero; PE ≡ PA(CH<sub>2</sub>)<sub>2</sub>NH<sub>3</sub> ≡ phosphorylethanolamine; PE(CH<sub>3</sub>) ≡ phosphoryl-N-methylethanolamine; PA(CH<sub>2</sub>)<sub>4</sub>NH<sub>3</sub> ≡ phosphorylbutanolamine; PE(CH<sub>3</sub>)<sub>3</sub> ≡ PA(CH<sub>2</sub>)<sub>2</sub>N(CH<sub>3</sub>)<sub>3</sub> ≡ PC ≡ phosphorylcholine; PA(CH<sub>2</sub>)<sub>4</sub>N(CH<sub>3</sub>)<sub>3</sub> ≡ phosphoryl-trimethylbutanolamine; PS ≡ phosphorylserine; PS(CH<sub>3</sub>) ≡ phosphorylserine methyl-ester. PA(CH<sub>2</sub>)(COH)H ≡ phosphorylethylene glycol; PA(CH<sub>2</sub>)(COH)<sub>2</sub>H ≡ PG ≡ phosphorylglycerol; PA(CH<sub>2</sub>)(COH)<sub>3</sub>H ≡ phosphorylerithriol; PA ≡ phosphoric acid; PA(CH<sub>2</sub>)H ≡ phosphoric acid methyl-ester; PA(CH<sub>2</sub>)<sub>2</sub>H ≡ phosphoric acid ethyl-ester; PA(CH<sub>2</sub>)<sub>4</sub>H ≡ phosphoric acid butyl-ester. For similar data pertaining to other lipids see [7, 26, 55].

<sup>(3)</sup> The relatively low transition temperature may be due to the tendency of this lipid to form system with tilted hydrocarbon chains at low pH.

<sup>(4)</sup> It is impossible to achieve complete protonation of PC, event at such low pH, in contrast to PE.

$(\Delta T_{m,h}^O = 2 \pm 0.5 \text{ K}) < (\Delta T_{m,cl} \sim 3.5 \text{ K}) \leq (\Delta T_{m,h}^H \leq 5 \text{ K}) \leq (\Delta T_{m,h}^{PO_4} \leq 9.5 \text{ K})$ , if the shift from the van der Waals interactions is neglected (2).

The above values are empirical. In order to clarify their origin at the molecular level it is necessary first to calculate contributions to the bilayer free energy change and its transition changes given in equation (2), by using some suitable, e.g. molecular force model of the polar membrane part. Subsequently, the corresponding chain-melting phase transition shifts must be evaluated by means of equation (1) and compared with the experimental values.

#### 4. Calculation of the free energy of the polar membrane region.

The electrostatic, coulombic contribution to the bilayer free energy can be obtained [6, 12] quite easily from standard diffuse double layer theory

$$G_{cl}(d_w) \simeq (16 \varepsilon \varepsilon_0 A N_A / \lambda) (kT / Ze)^2 \tanh^2 [Ze \psi_0(\lambda, \sigma) / kT] \coth (d_w / 2 \lambda) \quad (4)$$

as a function of the interlamellar water layer thickness,  $d_w$ , and the bilayer surface electrostatic potential,  $\psi_0$ . The latter can be further expressed in terms of the Debye screening length,  $\lambda = [\varepsilon \varepsilon_0 kT / 2000 N_A (Ze)^2 c]^{1/2}$ , the molecular lipid area  $A$ , and the net surface charge density,  $\sigma \equiv e/A$ , in the form

$$\psi_0(\lambda, \sigma) = (2 kT / Ze) \sinh^{-1} (Ze \sigma \lambda / 2 \varepsilon \varepsilon_0 kT). \quad (5)$$

Here  $c$  is the molar bulk salt concentration and other symbols have their usual meaning [13].

However, this contribution is not the most important one. Although the membrane electrostatic effects, such as the electrostatic phase transition shift, should in principle be screenable entirely by ions, the effect of lipid ionization is actually found not to be completely reversible by the addition of salt. This is because of the thermodynamic and structural consequences of the interfacial hydration which result from the lipid ionization; the latter effects are typically much greater than the direct electrostatic ones.

Experimentally, this is seen from the fact, for example, that, at least as far as the chain-melting phase transition shifts are concerned, the hydrational consequence of the phosphate-group protonation far exceeds the corresponding electrostatic phase transition shift being 9.5 K and 3.5 K, respectively. This demonstrates that the main contribution to the free energy of the polar membrane region of the membrane indeed comes from the lipid hydration,  $G_p \sim G_h \gg G_{cl}$ .

From a theoretical point of view the same can be concluded after having calculated the free energy of lipid hydration and its change at the chain melting phase transition and then comparing them with the corresponding electrostatic quantities.

Performing such a calculation is not an easy task, however, as no commonly accepted, ready-to-use prescription for the description of the bilayer hydration exists to date. Concluding from Monte-Carlo simulations, the orientation of water bound to the lipid layers follows the local electric-field orientation. This holds both for water molecules as a whole and for single water OH-bonds. Consequently, it may be concluded that the main trends in the lipid-water interaction energies can be discerned from electrostatic calculations [19].

I therefore use a nonlocal electrostatic model of hydration throughout this work. This differs from the standard electrostatic approach, phenomenologically speaking, in that it

(2) Distearoyl glycerophospholipids with several OH-residues per headgroup and in particular the corresponding phosphatidic acid and its alkyl-esters have unusually low chain melting transition temperature, possibly due to the chain tilt, which makes the analysis of the phase transition shifts non-trivial. Similar lipids with shorter chains reveal in this respect a much more simple pattern.

relies on the use of the *local* rather than of the *average* solvent and membrane properties ; for example, on the local excess rather than on the integral membrane density of the surface charge. This makes the model capable of dealing with the effects of water binding to the supramolecular surface and also allows for the consequences of direct intermolecular interactions, such as hydrogen bonds between the supramacromolecular constituents.

Let me exemplify the meaning of this latter difference for phospholipids. For such molecules the positive charge, on the one hand, is widely distributed over the hydrogens or methyl groups of the ammonium group and the nitrogen atom per se is nearly neutral. The negative charge, on the other hand, is associated with the oxygens of the phosphate, with the headgroup associated OH-groups, or with the carboxyl groups of the serine moiety. (It is noteworthy that owing to the atomic origin of such charges the precise molecular configuration or organization (monomer or lattice) influences only little the intramolecular charge distribution [20].) A typical headgroup part of the phospholipids thus consists of two « charged regions ». These are separated by a barrier which prevents intramolecular charge exchange and charge neutralization. Consequently, one may assume that a certain *local excess* charge exists on a given atom or group of atoms on each lipid molecule. The magnitude of this local excess charge,  $e_r$ , can be calculated from the appropriate quantum-mechanical models [20]. It can also be assigned some characteristics « charge density » value which is proportional to the accessibility of the atom or of the residue,  $\alpha_r$ , and inversely proportional to the corresponding surface area,  $A_r$ . Thus, in the first approximation the local density of the excess charge, on the one hand, is given by :  $\sigma_{p,r} = e_r(\alpha_r/A_r)$ , where the values of the parameters  $\alpha_r$  and  $A_r$  can be obtained, for example, from space-filling models. (Note that the ratio  $A_r/\alpha_r$  corresponds to the exposed surface area, which is available for the solvent or solute binding). On the other hand, the total *surface average of the local excess charge density* is obtained [25] by summing up the weighted single-contributions from all exposed residues

$$\sigma_p = \left[ A_{\text{surface}}^{-1} \times \sum_r A_r \right] \sum_r \sigma_{p,r} . \quad (6)$$

For mixed systems one may use the related phenomenological expression

$$\sigma_{p,\text{mix}} = \frac{\sum_{\text{lipid}} (\sigma_{p,\text{lipid}} A_{\text{lipid}})}{\sum_{\text{lipid}} A_{\text{lipid}}} . \quad (7)$$

When lipid headgroups interact in the absence of water, their effective basic « electroneutrality unit » consists of the nearest-neighbour charged groups in each headgroup lattice, most frequently of the phosphate and the ammonium groups, or of the phosphate or serine groups and their bound counterions. But polar residues can also form intermolecular hydrogen bonds along which charge-transfer inevitably occurs. In any realistic model of the lipid bilayer surface *intramolecular* as well as *intermolecular* charge neutralization must therefore be considered. In the present model this is achieved by considering solely such residues in the sum in equation (6) which are not involved as hydrogen donors and acceptors in the interlipid H-bonds.

In hydrated bilayers the situation is more complex as certain intermolecular hydrogen bonds may then be replaced by hydrogen bonds between the lipid and the water molecules. Moreover, even in the cases where no direct water-lipid hydrogen bonds exist, charge transfer between the lipid and water molecules takes place [21, 22]. For hydrated lipid bilayers (and certainly also for other hydrophilic macromolecular structures) the local excess charges are

therefore subject to extensive charge screening and charge-neutralization by the atomic local excess charges on the water molecules. This is partly a reflection of the immense efficiency with which the water molecules can participate in hydrogen bonds, but is also a consequence of the relatively large separation between the positive and negative residues and their associated atomic charges on a typical polar surface.

At least in principle the electrostatic dipolar, quadrupolar, and higher moments contribute to the hydrophilicity of the lipid headgroups as well. But experimental data presented in this and our previous works suggest that the direct effects of the surface dipoles and multipoles are far less important for the lipid hydration than the existence of the surface polar residues. This is probably chiefly owing to the screening of the headgroup associated charges by water which diminishes the range of dipolar and multipolar fields.

Taken together, a nonlocal electrostatic description of the bilayer hydration can be devised [23-25] which formally is analogous to the standard electrostatic double layer theory in that it accounts for the effects of the lipid-water association in a mean-field manner [23, 24] but which depends on the local rather than on the integral electrostatic membrane properties. Within the framework of such nonlocal electrostatic model the bilayer surface properties can be described in terms of the interfacial electrostatic and hydration potentials,  $\psi_0$ ,  $\psi_{h0}$ . The water-structure effects correspondingly are accounted for phenomenologically by means of a static and a high frequency dielectric constant,  $\varepsilon$  and  $\varepsilon_\infty = 2 - 6$ , respectively, and an apparent water-structure correlation length,  $0.07 < \xi < 3$  nm ; in the nonlinear approximation an effective « charge of the water molecules »,  $e_w \approx 3.5 \times 10^{-20}$  A s, which reflects the effects of interatomic charge-transfer in the hydrogen bond formation, is also needed [24, 25].

Expressed in terms of these parameters the bilayer hydration free energy is approximately given by

$$\begin{aligned} G_h(d_w) &\simeq - [16 \varepsilon_\infty \varepsilon_0 (1 - \varepsilon_\infty / \varepsilon) A N_A / \xi] (2 kT / e_w)^2 \\ &\quad \times \{ \tanh^2 [e_w \psi_0 / 4 kT] + \tanh^2 [e_w \psi_{h0} / 4 kT] \} \tanh (d_w / 2 \xi) \\ &\equiv G_h(\psi_0, \psi_{h0}) \tanh (d_w / 2 \xi) \end{aligned} \quad (8)$$

provided that the ionic screening length is much greater than the water-structure correlation length.

The electrostatic potential accounts for the « coulombic » part of the interfacial hydration. This arises because of structuring of water (below the saturation level) in the electric field associated with the net surface charge and can be expressed as

$$\psi_0 \equiv \psi_0(\xi, \sigma) = (2 kT / e_w) \sinh^{-1} (e_w \sigma \xi / 2 \varepsilon_0 \varepsilon_\infty kT)$$

in analogy with the expression 5 for the standard electrostatic surface potential  $\psi_0(\lambda, \sigma)$ .

The bilayer surface hydration potential describes the membrane capacity for direct water binding. It is in the first approximation given by :

$$\psi_{h0} = (2 kT / e_w) \sinh^{-1} [e_w (\sigma_p + \tau_p + \nu_p + \dots) \xi / 2 \varepsilon_0 \varepsilon_\infty kT] \quad (9)$$

where  $\tau_p$ ,  $\nu_p$ , ... are the surface densities of the dipolar, quadrupolar, etc., charge.

Fortunately, at least for lipids, the terms other than  $\sigma_p$  seem to be of minor importance, as has been already noted. The total hydration free energy of highly polar bilayers ( $\sigma_p > 0.2$  A s m<sup>-2</sup>), for which the hydration potential is relatively constant [25], may then be taken to a good approximation to be proportional to the excess surface charge density :  $G_h \propto \psi_{h0} \sigma_p \sim \text{constant } \sigma_p$ . Because of the interdependence between the shift of the chain melting phase transition temperature and the change of the (hydration) free energy at such

transition, the former is also approximately proportional to the (change of the) effective interfacial hydrophilicity parameter :  $\Delta T_{m,h} \propto \Delta G_{h,m} \propto G_{h,m} \propto \sigma_p$ .

Molecular and hydration parameters and the state of lipid bilayers all interdepend [27]. This causes the functional dependence of transition free energy change to be such that to a good approximation its relative variation is independent of the water content in the system :  $\Delta G_{p,m}(d_w)/\bar{G}_p \simeq \Delta G_{h,m}(d_w)/\bar{G}_h = \text{constant}$ . This causes the approximate relation :  $\Delta G_{p,m}(d_w) \simeq \Delta G_{h,m}(d_w) = \Delta G_{h,m} \tanh(d_w/2\xi)$ , also to be true. From previous expression and from equations (1-8) it then follows that the bilayer chain-melting temperature should vary with the interfacial separation as a hyperbolic tangent, as long as the lipid hydration is the main or only source of the free energy contribution  $G_p$ .

In fact, a water-dependence of the expected type is found experimentally for most lipids. Figure 1 shows that the chain-melting phase transition temperatures of several 1,2-dipalmitoyl-glycerophospholipids calculated from equations (1-8) agree quite closely with the corresponding experimental values. This supports the concept advocated in this and in our previous works that apart from the lipid chains the lipid hydration is the main determinant of the bilayer phase properties.

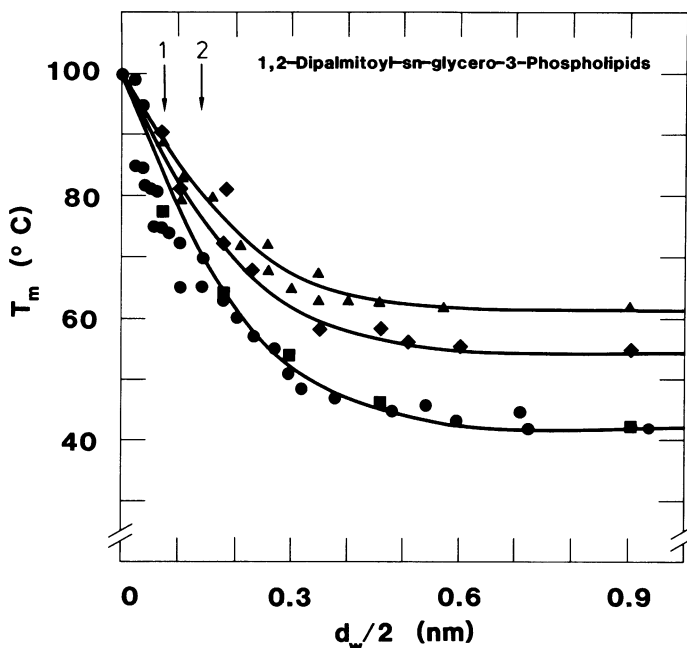


Fig. 1. — Measured (symbols correspond to the heating-run data) and calculated (curves) chain-melting phase transition temperature of 1,2-dipalmitoylglycerophospholipids : phosphatidylcholine (●) [10], phosphatidylglycerol. Na<sup>+</sup> (□), phosphatidylserine. Na<sup>+</sup> (◇), phosphatidic acid. Na<sup>+</sup> (▽), and phosphatidylethanolamine (Δ) [8] as a function of membrane hydration. The water layer thickness  $d_w$  for the experimental data was calculated from the known water/lipid molar ratio by assuming the molecular area to increase for these lipids upon hydration from 0.48 nm<sup>2</sup> to 0.70, 0.65 nm<sup>2</sup>, for the first two systems and from 0.38 nm<sup>2</sup> to 0.60, 0.55 and 0.55 nm<sup>2</sup>, for the other three. Arrows indicate approximate positions of mono- and di-hydrates. Curves were calculated by assuming  $\Delta S_{anh,m} = 157.5 \text{ J mol}^{-1} \text{ K}^{-1}$ , with  $\Delta G_{p,m} = \Delta G_{h,m} = 7.4, 7.4, 5.4$  and  $4 \text{ kJ mol}^{-1}$ . This is well within the range expected for these lipids from the estimated surface local excess charge density ( $\sigma_p$ ) and surface hydration potential values, using  $\xi = 0.25 \text{ nm}$  throughout.

The main phospholipid water-binding site and also the main centre of the surface local excess charge density is the phosphate group. By using the procedure described in previous sections a non-bound phosphate group can be estimated to give rise to  $\sigma_{p, PO_4^-} \approx -0.4 \text{ A s m}^{-2}$  [25]. The ammonium group is much less hydrophilic, not at least because some of its structural charge is typically involved in interlipid hydrogen bonding. The corresponding local excess charge density falls in the range  $\sigma_{p,r} \approx 0.11 - 0.18 \text{ A s m}^{-2}$ , depending on the degree of methylation [25]. Even less polar and less attractive for the water binding are the carboxylic groups and nonionic oxygens of the carbonyl or OH-residues.

These conclusions are in perfect agreement with the data of figure 2A. In this illustration the shift of the bilayer chain-melting phase transition temperature is shown as a function of the effective, total surface local excess charge density, used here as a measure of the effective bilayer surface hydrophilicity. The good correlation between the experimental data and the calculated curve supports the validity of the basic model assumptions and also of equation 7.

Experimental data on the variation of the bilayer chain-melting phase transition temperature as a function of the headgroup length are presented in table I. Whereas the chain melting phase transition of various alkyl-esters of phosphatidic acid vary only little with this parameter the transition temperatures of phosphatidylalkanolamines are strongly sensitive to the phosphorus-nitrogen separation in the neutral pH region, less so at low pH, and hardly at all in alkaline suspensions. Bilayers of diacyl- (or dialkyl-) glycerophosphoethanolamine or -butanolamine have different chain-melting phase transitions, in the case of eighteen carbon chains at 74 and 63 °C, respectively. In contrast to this, the corresponding lipids with trimethylated ammonium groups or « phosphatidyl-oligools », which all are incapable of forming comparably strong, direct interlipid bonds, have similar transition temperatures near 55 °C. This suggests that the headgroup length only influences the lipid phase behaviour significantly when it interferes with interlipid bond formation and thus with the lipid hydrophilicity.

Data presented in table I, moreover, indicate circumstantially that the dipole and higher multipole moments of the lipid headgroups (and consequently the parameters  $\tau_p$ ,  $\nu_p$ , etc.) indeed play only a secondary role in determining the lipid phase behaviour. If this was not the case, phosphatidylalkanolamines with long headgroups and large dipolar moments should be more hydrophilic and have a lower chain-melting phase transition temperature than the corresponding phosphatidic acid alcohol esters. In fact, however, even the electrostatically fully screened bilayers of methyl ester of phosphatidic acid bind water more strongly than phosphatidylethanolamine.

Also the dipolar fields emanating from the less exposed polar residues, such as the carbonyl groups of the acyl chains, are of little importance for the lipid hydration. This can be seen from the fact that the hydration of the acyl-chain carbonyl group results only in a small chain-melting phase transition shift of 1.5-2.5 K (data not shown). This is much lower than the  $5 \pm 1 \text{ K}$ -shift caused by the carboxyl-group hydration, despite the fact that the carbonyl groups of the fluid acyl-chains interact directly with the water [26]. The long-range, dipolar effects of the carbonyl groups are thus quite small.

The direct thermodynamic consequences of the lipid electrostatic moments are probably comparable to those which arise from the interlipid hydrogen bonds. The latter are difficult to model, however, because of their quantum-mechanical nature. Fortunately this does not severely hamper the present analysis. Firstly, owing to the fact that the resulting bond-induced transition temperature shift is quite small. And secondly because the knowledge of the interlipid bond-energy can alone serve as a basis for estimating the shift magnitude [17]:  $T_{m, \text{bond}} = \Delta E_{\text{bond}} / \Delta S_{\text{anh}, m}$ , the bonding energy  $E_{\text{bond}}$  being more easily available than the

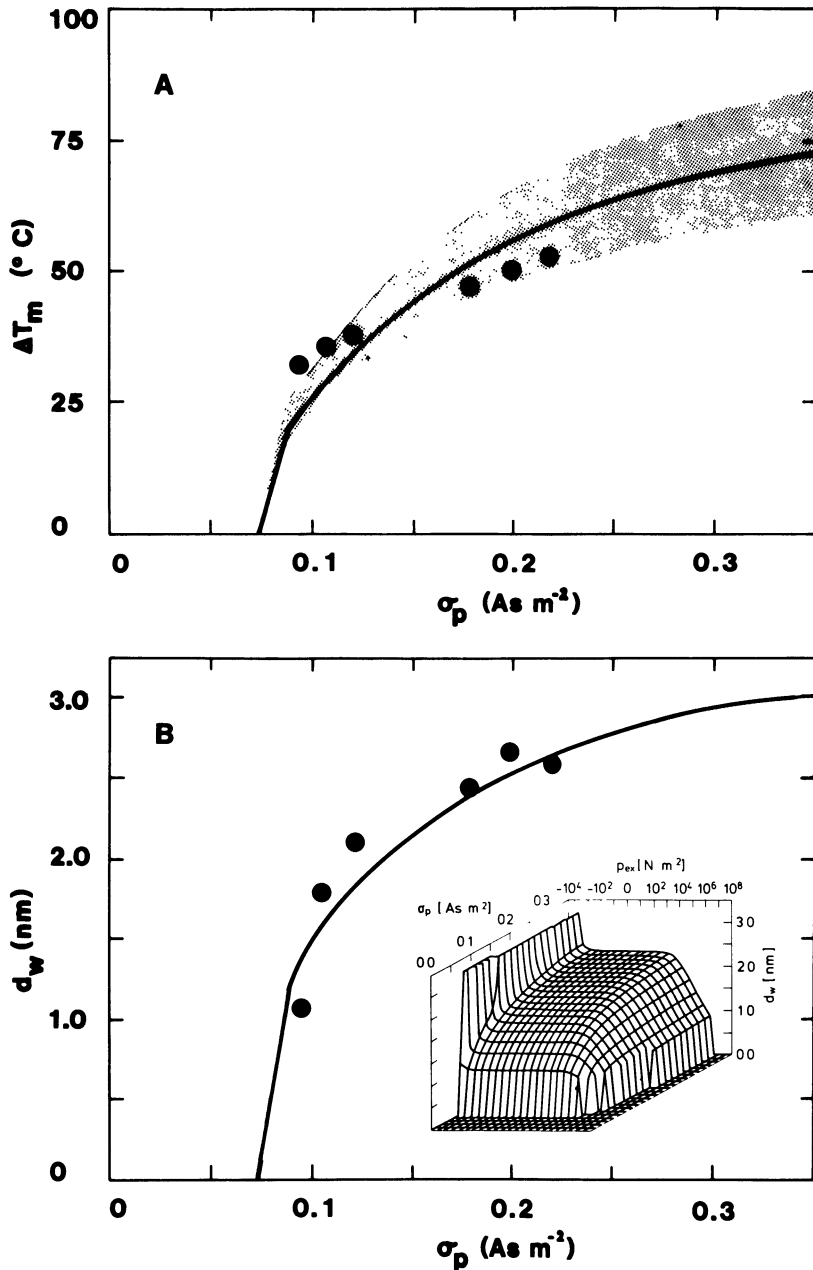


Fig. 2. — (A) The variation of the shift of bilayer chain-melting phase transition temperature,  $\Delta T_m$ , and (B) variation of the equilibrium interbilayer distance,  $d_w$ , with the effective surface local excess charge density  $\sigma_p$ , as a measure of the bilayer surface polarity. Experimental points were obtained with 1,2-ditetradecyl-phosphatidylethanolamines of various degree of headgroup methylation (PE through to PC) and their 1 : 1 mixtures. To calculate the curve in (B) a generalized theory of van der Waals forces which allows for the charge correlation effects [35] was used. The variation of the water structure correlation length was approximated by the phenomenological expression :  $\xi(d_w) = 0.073 \text{ nm} + 0.65 d_w$ , which is based on the optimal linear fit of the measured  $\xi(d_w)$ -data (see e.g. Ref. [6]). Insert to B : The effect of bilayer surface local excess charge density,  $\sigma_p$ , and the external pressure,  $p_{ex}$ , on the calculated value for the equilibrium interfacial separation  $d_w$ , as given by equation (11).

corresponding bond free energy <sup>(3)</sup>. Nevertheless, unless complex calculations are performed, the analysis of the bond-dependent bilayer phase transition shifts can to date only rely on the observation that H-bonded lipids have nearly identical transition temperatures in normal and in deuterated water. From this absence of the isotope effect it is concluded that energy of interlipid hydrogen bonds must be on the order of 10 kJ mol<sup>-1</sup>; from the shift value  $\Delta T_{\text{bond}} \approx 1 \pm 0.75$  K it is then calculated that the bond energy changes upon lipid chain melting by approximately 2%. More thorough discussion of the calculation of chain-melting phase transition shifts from the present theory is given in appendix A.

Finally, a general expression for the free energy from van der Waals interactions can be written in terms of the generalized Hamaker function  $H_A = H_A(d_w, \xi, \lambda)$

$$G_{\text{vdW}}(d_w) \approx AN_A H_A(d_w, \xi, \lambda) / 12 \pi d_w^2 \quad (10)$$

the value of the latter for small interfacial separations being greater than the normally assumed Hamaker constant of  $3 - 6 \times 10^{-21}$  J. This difference is a result of the solvent structure and interior correlation effects. Experiments (cf. Fig. 1) as well as the results of equation (10) suggest, however, that the shift of the bilayer chain-melting phase transition temperature which arises from van der Waals forces is normally negligible.

All of the theoretical results presented so far are reasonably accurate as long as the correlation length of water structure is comparable to or larger than the thickness of the polar interface. If this is not the case, the latter characteristic dimension begins to characterize the membrane hydration profile so that all system properties which are sensitive to hydration begin to depend crucially on the actual distribution of the surface polar residues. Owing to the interdependence between the interfacial separation and the bilayer structural parameters [25] the effective « decay length of the hydration force » under such conditions may become lipid- and hydration-dependent (to be published). This explains, among other things, the experimentally established dependence of the water-order correlation length on the degree of lipid methylation [36].

## 5. Molecular origin of the bilayer surface polarity and hydration.

Data presented in this work provide rather strong experimental evidence for the view that lipid hydration is predominantly of quantum-mechanical origin, rather than being a consequence of the surface dipoles, say.

This is seen, firstly, from the fact that the ratios of the partial chain-melting phase transition shifts and the corresponding surface local excess charge densities of the given lipid molecules are always of the same magnitude. Let me consider, for example, the phase transition measured with mixtures of two homologues of phosphatidylethanolamine of various degree of methylation (« 1 » and « 2 »), which are likely to have very similar dipolar moments. Experimental results are in good harmony with the theoretical predictions based on equations (1-7): if the effective surface local excess charge density is determined from (cf. Eq. (7)), this is, are taken to be given by  $\sigma_{p,ef} = (\sigma_1 A_1 + \sigma_2 A_2) / (A_1 + A_2)$ , the agreement is nearly quantitative (Fig. 2A).

Secondly, the chain-melting phase transition shifts elicited by the monomethylation of the carboxyl group of phosphatidylserine or of the ammonium group of phosphati-

<sup>(3)</sup> By simple thermodynamic arguments it can be demonstrated that the entropic and enthalpic parts of the free energy  $G_p$  from bond breaking cancel [17] so that the corresponding contribution to  $\Delta G_{p,t}$  is just  $\Delta E_{\text{bond},t}$ , i.e. is identical to the change in the bond-strength at  $T_t$ . The phase transition temperature shift  $T_{m,bond}$  is then proportional to the latter.

dylethanolamine are identical (cf. Tab. I), as are also the corresponding estimated changes of the surface local excess charge density. Thirdly, the chain-melting phase transition temperatures of either uncharged phosphatidylserine and monomethylated phosphatidylethanolamine, or of electrostatically screened phosphatidylglycerol and dimethylated phosphatidylethanolamine, are in excess solution very much the same. So are the corresponding surface local excess charge densities for each of these lipid pairs :  $\sigma_{p, PS} \approx \sigma_{p, PE(CH_3)}$  and  $\sigma_{p, PE(CH_3)_2} \approx \sigma_{p, PG}$ . And lastly, the absolute values of the quotients  $\Delta T_{m,h}^{PO_4} / \Delta T_{m,h}^H \approx 2.75$  and  $\sigma_{p,O} / \sigma_{p,-O} \approx 2.5$  or of  $\Delta T_{m,h}^{COO} / \Delta T_{m,h}^H \approx 1$  and  $\sigma_{p,COO} / \sigma_{p,H} \approx 1$ , also are always similar in accordance with the predictions of the model introduced in this work.

This suggests that lipid hydration is chiefly a consequence of direct water binding to the surface polar residues and, indirectly, of the coupling between the water molecules.

### 6. Effect of surface polarity on the interfacial separation and lipid fusogenicity.

At low pH, where the molecular hydration potential is typically the lowest, vesicles made of any common phospholipid flocculate or precipitate rapidly. This effect of the protonation of anionic oxygens is not prevented by the net electric charge on the ammonium group. Protonation induced changes are also frequently accompanied by gross bilayer fusion, as seen from gel chromatography and optical density measurements (data not shown). Moreover, at very low pH below the pK-value of the phosphate group, ( $pH < pK_{PO_4} < 1 - 2$ ) bilayers of phosphatidic acid, phosphatidylethanolamine and phosphatidylserine readily partly dehydrate and form new phases. The latter may have tilted chains and consequently low chain-melting transition temperatures which for the lipids with eighteen carbon atoms per chain is often around  $T_{m,anh} \sim 75^\circ C$  (data not shown).

At  $pH = 8$  the colloidal stability of lipid suspensions is larger than at low pH. Notwithstanding this, bilayers with relatively low hydration potential and correspondingly high chain-melting phase transition temperature still tend to lose part of their hydration in the neutral pH-region if they are incubated below or not much above the chain melting transition temperature [28]. Significantly, the rate of such dehydration is greater for the less polar lipids.

At  $pH > 12$ , finally, vesicles of any chemically stable glycerophospholipids in moderately concentrated salt solutions stay as stable dispersions indefinitely. Under these conditions their hydration potentials are also the highest.

To clarify these phenomena and correlate the colloidal and phase behaviour of lipid vesicles I have studied the sum of the water and lipid layer thickness, i.e. the measured lamellar repeat distance, as a function of the lipid headgroup and bulk pH-value (Tab. II). In the neutral pH-region the water layer thickness estimated from these values was found to increase with the interfacial hydrophilicity (expressed in terms of the surface local excess charge density parameter  $\sigma_p$ ) both below and above the chain-melting phase transition temperature.

The situation at low pH is experimentally less clear. Nonetheless, the values pertaining to low pH, where most of the membranes bear a net positive charge (of somewhat variable surface charge density), indicate that the net electrostatic charge is unlikely to dominate the interbilayer interactions under such conditions. If this was the case, in the acidic suspensions this charge would lead to indefinite swelling of the lipid aggregates as it does for anionic bilayers at high pH (Tab. II). Experimental data on ion- or pH-induced fusion between macromolecular and supramolecular assemblies consequently should never be analyzed solely in terms of surface electrostatics and the effects of changing the membrane hydration should always be considered. The parallelism between the pH-dependence of the lipid colloidal stability, the bilayer phase behaviour, and the interfacial hydrophilicity suggests that neither of these is regulated chiefly by the bilayer surface electrostatics but rather are primarily a

Table II. — *Interlamellar repeat separation* (nm) <sup>(1)</sup> *and estimated interbilayer water layer thickness* (*italics* (nm)) <sup>(2)</sup> *as a function of headgroup type and degree of protonation for two relative temperatures.*

Lipid <sup>(3)</sup>	pH = 0 <sup>(4)</sup>		pH = 7			pH = 13				
	<i>T<sub>m</sub>-10 K</i>	<i>T<sub>m</sub>+10 K</i>	<i>T<sub>m</sub>-5 K</i>	<i>T<sub>m</sub>+10 K</i>	<i>T<sub>m</sub>-15 K</i>	<i>T<sub>m</sub>+10 K</i>				
DTPE(CH <sub>3</sub> ) <sub>3</sub>	6.06	5.65	1.85	6.25	1.63	6.20	2.62	6.09 <sup>(5)</sup>	6.18	2.50
DTPE(CH <sub>3</sub> ) <sub>2</sub>	6.25	5.75	1.80	6.19	1.57	6.12	2.50	> 11 <sup>(6)</sup>	> 11	> 7.3
DTPE(CH <sub>3</sub> )	6.30	60.8	2.30	6.42	1.35	5.88	2.18	> 11	> 11	> 7.3
DTPE	6.14	5.90	2.22	5.50	0.4	4.90	1.10	ND	ND	ND

<sup>(1)</sup> X-ray diffraction measurements were performed using a Guinier camera with a bent quartz crystal monochromator isolating the CuK<sub>α1</sub>-line. Exposure times were 15-30 min, temperature accuracy  $\pm 1$  K.

<sup>(2)</sup> In the evaluation of  $d_w$  from the measured repeat distance for  $T < T_m$  the tilt angle of phosphatidylcholine was assumed to be the same at pH = 8 and > 12 : 36°. The tilt of dimethylated phosphatidylethanolamine was taken to be 32°, to conform with that of phosphatidylglycerol [56], since these two lipids have similar hydration potentials. The water layer thickness for  $T > T_m$  was obtained from the repeat separation by assuming that the lipid layer thickness is 3.7 nm for phosphatidylethanolamine and its monomethylated derivative and 3.8 nm for dimethylphosphatidylethanolamine and phosphatidylcholine, respectively. For the theoretical values of  $d_w$  see figure 2.

<sup>(3)</sup> DT  $\equiv$  1,2-ditetradecyl-sn-glycero ; for other abbreviations see legend to table I.

<sup>(4)</sup> Because of the different pK-values of PC and PE the degree of headgroup protonation and thus the net surface charge density are not yet maximal at this pH.

<sup>(5)</sup> Phosphatidylcholine at this pH is still zwitterionic.

<sup>(6)</sup> Our x-ray camera did not allow for measurement of repeat distances > 11 nm (ND  $\equiv$  not determined).

function of the interfacial polarity and hydration. In other words, the increased lipid colloidal and fluid phase stability both seem to be a consequence mainly of the increased lipid hydration and less so of electrostatic potential.

The reason for this is that only the long-range interbilayer interactions depend to any significant amount on the ordinary membrane electrostatics [30-32]. Phenomena such as membrane adhesion and fusion, which are sensitive mainly to the short-range forces, conversely, are governed by the counterplay of the hydration and (generalized) van der Waals forces [33-35]. Coulombic charges, therefore, may regulate the association between lipid vesicles but it is primarily the surface local excess charges and the interfacial hydrophilicity which control the fusion capacity of lipid bilayers.

This conclusion can be substantiated by inspection of the theoretical dependence of equilibrium membrane separation,  $d_w$ , upon changing the bilayer hydration potential and pressure imposed on bilayers. If the distance between membranes is determined from equations (4-10) by requiring that the free energy of the system at equilibrium should be minimum, i.e. by setting the first derivative of the free energy with respect to the separation to be zero, the following equation is found

$$d_w = 2 \xi \cosh^{-1} \{ 2 [p_{ex} + p_f + H'_A(d_w, \xi)] \xi / G_h(\psi_0, \psi_{h0}) \}^{1/2}. \quad (11)$$

The bilayer potentials  $\psi_0$  and  $\psi_{h0}$  and the bilayer hydration self-free energy  $G_h$  are defined in

the previous section. The pressure  $p_{\text{ex}}$  incorporates the external hydrostatic and osmotic, or the internal electrostatic pressures ; numerically these are identical to the corresponding partial derivatives of the free energy.

In addition to these, an extra pressure contribution has been introduced into equation (11) : the interfacial repulsion arising from the surface fluctuations [37, 38]. The latter can be evaluated as :  $p_f = (\pi kT/32)(\partial^3 G_p(d_w)/\partial d_w^3)[BAN_A(\partial^2 G_p(d_w)/\partial d_w^2)]^{-\frac{1}{2}}$  [37], where the free energy  $G_p$  and its derivatives can be found from equation (2).

The parameter  $B$  in equation 11 is the elastic curvature modulus ; for lipid bilayers it is typically on the order of  $10^{-19}$  J. The spatial variation of the Hamaker function required in equation 11 can be estimated from a generalized semi-quantitative theory of van der Waals forces (4). This accounts explicitly both for the attraction from dispersion interactions, as well as from the hydration and charge correlation effects [35]. The water-order correlation length hereby is assumed to vary approximately linearly with the interfacial separation. (A theoretical rationale for such dependence in the region of low hydration will be given elsewhere).

The solutions to equation (11) are given in figure 2B as a function of the surface local excess charge density,  $\sigma_p$ , as a measure of the surface hydrophilicity, and the pressure imposed on bilayers,  $p_{\text{ex}}$ . They show that for relatively apolar surfaces the interacting membranes are likely to be relatively tightly opposed. For slightly more hydrophilic surfaces ( $\sigma_p \geq 0.07 \text{ A s m}^{-2}$ ), characterized by somewhat higher values of the interfacial hydration potential, the equilibrium interfacial separation should then increase relatively steeply with the surface local excess charge density. Ultimately, however, the distance between interacting surfaces should become rather insensitive to further variations of the surface local excess charge density because of the saturation-of-hydration effect. The latter is similar in nature to the saturation-of-ion-density in the diffuse double layer region which is predicted by classical electrostatics. Mathematically it is reflected in the levelling-off of the « sinh »-function, which describes the dependence of the interfacial hydration potential on the local excess charge density  $\sigma_p$ . One experimental manifestation of this is the strong effect of the first methyl group on the lipid headgroup.

Correspondingly, starting with highly hydrophilic, strongly hydrated bilayers and lowering the bilayer hydration potential should initially affect the equilibrium interfacial separation only slightly. However, by decreasing the effective value of the parameter  $\sigma_p$  below a certain threshold value, for example by lipid protonation, demethylation, or ion binding, should then induce a rather sudden colloidal collapse of the lipid vesicles — even for excess solution. Such collapse may also lead to a (partial) bilayer dehydration or fusion (see insert to Fig. 2B).

This is also found experimentally upon adding to the lipid suspension ions with a high surface charge density, such as  $\text{H}^+$ ,  $\text{Li}^+$ , or di- and polyvalent ions [28, 29, 39-42]. The reason for this is the competition of such ions with water molecules for the same binding sites on the polar residues [43]. Owing to the fact that such occupied residues no longer can contribute to

(4) To obtain an analytical approximation, the function  $H_A(d_w, \xi)$  is separated in two parts. The first one, originating from standard van der Waals interactions, becomes smaller at small interfacial separations than the classical dispersion force model predicts, because of the water-structure effect. The second is due to the correlations between the (local excess) charges on the adjacent bilayer surfaces ; it is amplified by the water structure and causes the total interfacial attraction to be greater at small interfacial separations than the result obtained for a constant value of the Hamaker function,  $H_A(d_w, \xi) = H_A = \text{constant}$ , this latter contribution for closely apposed surfaces being by more than one order of magnitude greater than the former, as can be seen from the phenomenological expansion :  $H'_A(d_w, \xi) \equiv \partial H_A(d_w, \xi)/\partial d_w = H'_A(d_w) = H_A(0.052131 + 0.209808 d_w - 0.0142705 d_w^2 + 0.0002761 d_w^3)$ .

the surface polarity the parameter  $\sigma_p$  decrease and causes the bilayer hydration potential to become smaller. As a result of this the membranes are less attractive to the solvent. The ion-induced lowering of the lipid « solubility » in water, which is implied by the present model, is also directly observed with aqueous solutions of (chainless) lipid headgroups [44] <sup>(5)</sup>.

### 7. The role of interfacial hydrophilicity in biological systems.

So far the discussion has been centred on lipid bilayers. However, the concepts of this work may be applied to any solvated system, from biomolecules in solution through to cells involved in recognition processes. A few examples of experimental observations which can be explained on the basis of the principles introduced in this work are as follows.

DNA-strands with major hydration sites on the phosphate and sugar groups exhibit colloidal behaviour closely resembling that of lipid bilayers [47, 48]. Curves of the interstrand force, for example, are found to be displaced towards shorter separations if the ion charge density is increased, and double helical DNA precipitates from solution upon addition of trivalent ions or multivalent biological polyamines [48]. Moreover, at short separations an anomalously large attractive force is found similar to that implied in this study to occur with phospholipid bilayers.

The rate of synthesis of pyrophosphate from  $P_i$  in the presence of yeast pyrophosphatase increases if the pH-value of the solution is lowered, if divalent ions or non-aqueous solvent are added in the medium, or if an osmotic stress is imposed on the samples, presumably all because such treatments induce the condensation of the phosphate on the active enzyme site [49]. Similar conclusions are likely to apply to other enzymes involved in the mechanism of energy transduction in living cells.

Viral proteins typically enter cells or fuse with liposomes only after a pH-drop [50, 51], the efficiency of the latter process being dramatically reduced by exchanging phosphatidylethanolamine for phosphatidylcholine, which increases the bilayer hydrophilicity; diphtheria toxin becomes hydrophobic and toxic only in acidic environments where the molecule is partly protonated [52]; thylakoid membranes of the higher plant chloroplasts stack upon increasing the concentration of  $Na^+$  or  $H^+$  ions in the solution [53]; the antibody titer against small haptens embedded in lipid membranes is significantly lower for bilayers of higher surface polarity, which exhibit stronger interfacial repulsion [54]; many further examples could be cited.

### 8. Conclusions.

Data presented in this work indicate that the phase and colloidal behaviour of biomolecules, here illustrated with the cases of the bilayer ordered-phase stability, of interactions between lipid bilayers, and of vesicle fusion, are all closely related. They are determined primarily by the quantum-mechanical interactions between water and amphiphiles, i.e. by the hydrophilicity of the polar part of the molecule. Using a molecular force hydration theory it is found, for example, that the same choice of the bilayer hydration potential, as a measure of the lipid hydrophilicity, that ensures agreement between the measured and calculated transition temperatures also provides good estimates of the interlamellar water layer thickness. Changes

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<sup>(5)</sup> In this context it is important to realize that interfacial ions not only decrease the lipid hydrophilicity, but moreover give rise to extra interfacial attraction, owing to their mutual correlations. This attraction increases with the charge density of the adsorbed ions [45] and with lowering of the interfacial dielectric constant [35, 46] and is likely to be more important for the stability of lipid vesicles than actual interbilayer salt-bridges, except perhaps for bilayers in close contact.

in the lateral packing density of lipid bilayers and in the interfacial layer thickness can also be explained along the same lines. This clarifies the effect of the polar region of the membrane on the phase and colligative properties of all common glycerophospholipids. Moreover, it provides a rationale for developing theoretical models for the description of other biologically relevant systems, such as DNA-strands, viral capsules, etc.

The bilayer properties can be influenced from the point of the polar region of the membrane by either chemically modifying (e.g. methylating) the lipid headgroups, or by changing the properties of the latter physicochemically (e.g. by varying the solvent composition). The main reason for this is that the lipid/ion association not only affects the bilayer electrostatics, as is often believed, but that it also and more importantly changes the bilayer hydration; the reason for the latter change is the modification of the lipid headgroup capacity to bind water. The effects of interlipid bonds and dispersion forces on the bilayer chain-melting transition temperature appear to be quite small. However, the generalized van der Waals forces, which include the charge-correlation effects, are quite important for the colloidal behaviour of molecular aggregates, especially in the case of closely apposed bilayers.

### Acknowledgments.

I wish to thank J. M. Seddon for critically reading the typescript and am indebted to G. Blume for assistance in the synthesis of some lipids. Parts of this study have been supported financially by the Deutsche Forschungsgemeinschaft.

### Appendix A.

If the magnitudes of single contributions to the membrane free energy and their changes at the phase transition are known, the resulting phase transition temperature shifts can be determined from equation (1).

The purely electrostatic part of the bilayer free energy can be found from standard diffuse double layer theory of Gouy and Chapman as a function of the surface charge density. The result for bilayers consisting of singly charged phospholipids is that the electrostatic free energy should be on the order of  $10 \text{ kJ mol}^{-1}$  or less.

This bilayer free energy must decrease at the chain-melting phase transition because of the concomitant increase of the molecular lipid area. Concluding from x-ray diffraction data [14-18], the relative area increase at the phase transition is:  $0.05 < (\Delta A_m / \bar{A}) < 0.3$ ; the smaller values typically correspond to systems with larger values of the area  $\bar{A}$ , and *vice versa*. Based on such estimates it is found from equation 4 that for singly charged lipids in a 0.1 molar 1:1 salt solution with large molecular areas on the order of  $0.75 \text{ nm}^2$  one should have,  $\Delta G_{\text{el},m} \approx 12(\Delta A_m / \bar{A}) \text{ kJ mol}^{-1} \approx 0.6 - 1.2 \text{ kJ mol}^{-1}$ . This corresponds approximately to  $3 < \Delta T_{\text{el},m} < 6.5 \text{ K}$  for the lipids with eighteen carbon atoms per fully saturated chain, a value which is not too different from the experimental estimate  $\Delta T_{\text{el},m} \approx 3.5$ . However, it should be noted that around 50 % of this is due to the « coulombic hydration » (see the text), that is, it stems from the potential-dependent term in the expression for the bilayer hydration free energy (see Eq. (8)). The electrostatic shift arising from the second charge on a lipid molecule is even smaller,  $\Delta T_{\text{el},m}^{+,+,-,-} - \Delta T_{\text{el},m}^{+,-} < 2 \text{ K}$ . (The « indirect » thermodynamic consequences of lipid ionization are thus at least as important as the direct ones: the elimination of the net charge from a polar residue nearly abolishes the variation of the bilayer chain-melting phase transition with the number of protons on this residue (cf. Tab. I), because it strongly diminishes the H-bonding capacity of the residue.)

The bilayer hydration free energy can be evaluated from the nonlocal electrostatic

membrane theory (cf. Equations (8), (9)) in terms of the surface polarity parameters  $\sigma_p$ ,  $\tau_p$ , etc.

The main contribution is the surface local excess charge density,  $\sigma_p$ . This is also the chief source of the membrane hydration and for phospholipids originates mainly from the phosphate group, chiefly due to the affinity of its anionic oxygens for water. The excess charge density estimated from equation (6) for such oxygen ions is  $\sigma_{p,O} \approx -0.18 \pm 0.02 \text{ A s m}^{-2}$ . This is much greater than the corresponding value pertaining to ester oxygens,  $\sigma_{p,-O-} \approx -0.04 \text{ A s m}^{-2}$  [25, 26]. Nonetheless, if the latter are numerous they can significantly contribute to the overall lipid capacity for water binding. This can be seen, for example, in the data given in table I for the series of phosphatidylglycerol analogues with varying number of hydroxyl groups.

The entire phosphate group gives rise to a total local excess charge density of  $\sigma_{p,PO_4} \approx -0.4 \text{ A s m}^{-2}$ . This is much higher than the corresponding value for the ammonium or trimethylammonium groups:  $\sigma_p \approx 0.18$  and  $0.11 \text{ A s m}^{-2}$ , respectively [26]. The ammonium group therefore binds water much less efficiently than the phosphate, the difference being even greater when this groups is involved in intermolecular hydrogen bonds. Despite this, on the average, it can bind water nearly twice as strongly as a single carboxyl group or a headgroup associated proton. In harmony with this from equation (6) the latter two residues are estimated to be characterized by relatively low effective local excess charge densities,  $\sigma_{p,COOH} \approx -0.07 \text{ A s m}^{-2}$  and  $\sigma_{p,H} \approx 0.07 \pm 0.006 \text{ A s m}^{-2}$ , respectively [25].

Using the above values together with equation (8) and considering that the total surface density of the local excess charge is smaller than the corresponding densities for the single sites or residues by a factor  $\left( \sum_r A_r \times A_{\text{surface}}^{-1} \right)$  (cf. Eq. (7)) one gets for the bilayer hydration free energy values between 2 and 15  $\text{kJ mol}^{-1}$ . Upon chain fluidization this value can become more negative by as much as 50 % [25], because of the increased accessibility of the surface polar residues to the water molecules. It is noteworthy, however, that this effect is smaller for the more hydrated bilayers owing to the saturation of the hydration effect. The smaller is therefore the hydration free energy, the greater the is its change at the phase transition.

From all this, it may be concluded that the hydration induced chain-melting phase transition temperature shift should be 40 K or less for the lipids with eighteen methyl(ene) groups in each saturated hydrocarbon chain. The shift arising from the carboxyl group protonation or from the change in the number of headgroup-associated protons by one, according to the present theory, should amount to approximately 12 % of this value. The measured ratio is 1/0.125.

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