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Polymorphism of the bilayer membranes in the ordered phase and the molecular origin of the lipid pretransition and rippled lamellae

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The lamellar-to-undulated-lamellar phase transition ($L_{\beta'} \rightarrow P_{\beta'}$, pretransition) in lipid bilayers is shown to be a phenomenon which exists only if lipid polar headgroups are sufficiently hydrated and if the interchain packing is sufficiently weak. The minimal lipid hydrophilicity and the critical amount of the lipid-bound water can be related to the lipid chain-melting transition temperature; the latter must not exceed some maximal, chainlength-dependent value if the pretransition is to exist. The minimally required amount of the lipid-bound water itself is essentially chainlength independent, however, and unaffected by the method of hydration variation: physical dehydration, hydrational competition between the lipid molecules and the substances dissolved in the aqueous subphase, or decreasing lipid headgroup polarity all affect the pretransition temperature similarly on the appropriate scale. Simple, phenomenological expressions for the evaluation of the bilayer subtransition, pretransition and chain-melting phase transition temperature as a function of the lipid chainlength are presented. They show that, even in excess water, bilayers will tend to undulate only as long as each of the two identical lipid chains will contain between 12 ± 1 and 22 ± 1 carbon atoms; the $P_{\beta'}$-phase region for the less polar lipids being as a rule narrower. To get a theoretical means for quantitatively studying the effects of the lipid hydration on the bilayer pretransition, an interaction-balance method is proposed for describing undulated membranes at the molecular level. This is based on comparing the free-energy gain from the increased headgroup hydration with the free-energy loss caused by the reduced chain-chain attraction upon ripple formation. A rationale is thus found for scaling the pretransition temperature in terms of the hydration-induced chain-melting phase transition shift or of the lipid surface hydrophilicity (see Fig. 8). Within the framework of such a model the recently reported (de)hydration dependence of the bilayer-undulation period is reproduced with reasonable accuracy. Furthermore, it is estimated that at least 12 ± 2 water molecules must be associated with each lipid head for the bilayer undulation to be feasible. The closer the system is to this boundary condition the longer is the repeat-distance for the surface undulations and the less stable is the undulated bilayer phase.

Introduction

Phospholipids undergo a variety of thermotropic and lyotropic phase transitions. Of these, the so-called pretransition, which is a lamellar-to-periodic gel ($L_{\beta'} \rightarrow P_{\beta'}$ or $smB_C \rightarrow smB_{CA}$) transition, is the least well understood despite the fact that it is experimentally well documented and has also received extensive theoretical discussions. In this work I present new experimental data which highlight some of the previously unknown or underestimated facts pertaining to such pretransition in phospholipid bilayers. In particular, I discuss the range of existence and clarify the conditions for the formation of the lipid pretransition. I also inspect in detail the origin of the pretransition in lipid bilayers on the basis of a model which I have briefly introduced previously [8]. Finally, I interpret certain structural parameters of the rippled bilayers at the molecular level.

Hydrated lipids at relatively low temperatures typically form densely packed crystalline structures [26,30,41] (Fig. 1). Most researchers believe that hydrocarbon chains in such structures form an ortho-rhombic hybrid chain-subcell lattice [26,30,17,1]. With the in-

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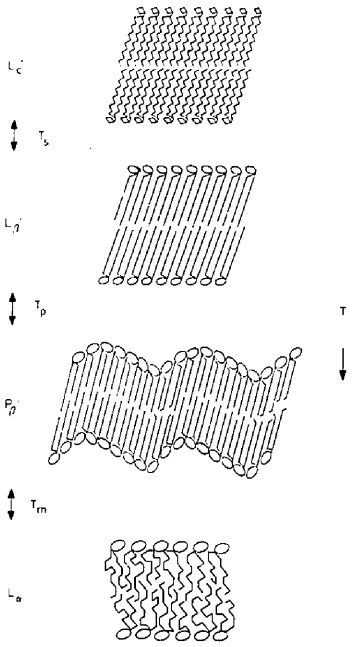

Fig. 1. Schematic representation of the lipid polymorphism in the gel phase. The illustrated sequence is believed to represent various phases of phosphatidylcholine/water mixtures in the ordered phase.

creasing temperature the hydrated lipid crystals become energetically unfavourable owing to the thermal, initially chiefly rotational, chain excitations. In consequence, at a certain *subtransition* temperature, $T = T_s$, such two-dimensional crystals revert into a more expanded lipid-gel phase of the β- or, more frequently, β'-type [38,11]. Lipid chains in the gel state are packed on a hexagonal lattice and are moderately tilted ($L_{\beta'}$ [18]) or untilted (L_β [26]). The properties of the chains in such phases resemble those of the long-chain hydrocarbons, such as alkanes, above the rotator phase transition [32].

Heating lipids above the subtransition temperature speeds up oscillations of the hydrocarbon chains. This terminates in an essentially unhindered, long-axis chain rotation [15,39] often in a cooperative manner at the *pretransition* temperature, $T = T_p$. The lipid headgroup mobility, most notably the rotation of the lipid headgroups around the P-O-bond to the glycerol backbone [36], as well as the interfacial area per molecule consequently increase substantially at the pretransition. The area per each phosphatidylcholine headgroup, for example, below and above the pretransition temperature is believed to be 0.525 nm^2 and 0.65 nm^2, respectively [37,28], the concomitant change in the chain area being only a few percent. It is very probable that during the pretransition individual chains mutually shift along their long axes to stay in close contact; this is likely to be the reason why the bilayer surface breaks up into a series of periodic, asymmetric, quasi-lamellar bilayer segments which give rise to the appearance of the surface undulations or ripples characteristic of the $P_{\beta'}$ or P_β phase [42]; in the older literature sinusoidal waves are discussed as a possible ripple-form.

In the low-temperature, gel phases the hydrocarbon chains are in an orientationally well-ordered state in which the hydrophobic molecular segments are essentially in an all-*trans* configuration; their extension is thus close to the maximum possible value (see, for example, Refs. 30 and 38). At higher temperatures, however, the high chain-order is lost, owing to the orientational chain-excitations, resulting in a cooperative chain-melting (order-disorder, gel-to-fluid) phase transition at $T = T_m$. One possible sequence of the lipid phase transitions in the gel phase is summarized in Fig. 1.

Being determined largely by the chains, the subtransition temperature is relatively insensitive to the water concentration in the system. It also depends little on the concentration of solutes in the aqueous subphase. The initial effect of changing hydration on the lipid chain-melting phase transition temperature is relatively small as well. Upon moderately decreasing water content or strongly increasing the bulk salt concentration in the system the chain-melting phase transition temperature is changed by less than two percent (less than 5 degrees) as long as the headgroup protonation remains the same. In general, however, hydration is an important parameter of the lipid polymorphism. Extensive dehydration can cause phase transition shifts on the order of 50 degrees and more. Lipid chain-melting phase transition therefore acts as a thermodynamic osmometer: the chain-melting phase transition shift is directly proportional to the change of the logarithm of the *bulk* water activity coefficient [9], the sensitivity decreasing with the nominal hydrocarbon chain length.

Lipid pretransition is somewhat different in this respect. It reacts in similar manner but behaves as a strongly *interface*-biased osmometer, as argued in this work. The sensitivity of such 'pretransition-osmometer' may be quite high: for sufficiently hydrated surfaces even a small decrease of the interfacial water activity or a moderate increase of the interfacial solute concentration will shift the pretransition temperature appreciably, most frequently in the upward direction. The magnitude of such a solvent-dependent shift decreases with the lipid chainlength but is always much greater than the shift of the order-disorder phase transition, owing to the smaller enthalpy of the pretransition compared to the chain-melting enthalpy.

In this work I present experimental data which point to one possible explanation of such effects and, moreover, define the range of existence of the undulated phospholipid bilayers.

Materials and Methods

For all experiments suspensions of multilamellar or oligolamellar vesicles were used. Such vesicles were prepared by solvating various finely powdered diacylphosphatidylcholines (PC) or the appropriate mixtures of phosphatidylcholine-dimethylphosphatidylethanolamine homologs in an aqueous solution or in water by vigorous vortex mixing or mild ultrasonication, respectively. Phosphatidylcholines were purchased either from Boehringer (Mannheim, F.R.G.) or from Avanti Biochemicals (Birmingham, AL, U.S.A.); 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-(*N*,*N*)-dimethyl ($DMPE(CH_3)_2$) was a product of Laradon Fine Chemicals (Malmö, Sweden) or was made in our laboratory as described previously [33]. All lipids were confirmed prior and after the experiments to be more than 99% pure by means of thin-layer chromatography using chloroform/methanol/water (65 : 35 : 5, v/v) as a solvent and molybdenum blue staining followed by sulfuric acid charring for the detection. Water was doubly distilled in an all-glass apparatus. All salts (Merck, Darmstadt, F.R.G.) were of p.a. grade. Electrolyte solutions typically contained no buffering substance, but use of triethanolamine (pH 7.5) did not affect the results.

For the hydration studies, the lipids were dried under vacuum (< 10 Pa) over phosphorus pentoxide for several days. The water content in the sealed stainless-steel calorimetry pans after the addition of a small volume of H_2O was determined by weighing.

For the experiments with aqueous solutions, dry lipids were mixed with at least 500-fold excess (weight/weight) of suspending solution containing appropriate solutes. Only occasionally, intermediate concentration points were prepared by mixing two ready-to-use lipid suspensions and sample-aging for at least a weak at room temperature; they normally fitted nicely on the experimental curves.

In one set of experiments with saccharose, different sugar solutions with appropriate carbohydrate concentration were used to solvate the lipid. In another set, corresponding amounts of sugar were weighed into a pre-prepared vesicle suspensions and measured after one day of aging (cf. Fig. 9).

For the calorimetric experiments, lipid material was collected from the dilute vesicle suspension in mixing glass vials by a centrifugation.

To experimentally determine the phase transition temperatures the temperature variation of the optical density, of the bilayer accessibility to various labeled compounds, or of the specific enthalpy-change were measured on a Beckmann UV-vis spectrometer, on a Perkin-Elmer LS-5 fluorimeter, or on a Perkin-Elmer DSC-2 differential scanning calorimeter equipped with an Intracooler, respectivley. Typical temperature-scan rates were 1–2.5 degrees per min. Quoted transition temperatures correspond (unless stated otherwise) to the point at which upon heating the maximum of sudden, reproducible change in the measured parameter, or an onset of the calorimetric transition is observed. All results are means of at least four independent experiments.

Results

The chainlength dependence of the phase behaviour of fully saturated, symmetrical chain phosphatidylcholines in water has been investigated extensively for years. Ample data are available [20,1,30,26,27,22]. A complete set for the lipids with ten and up to twenty-two carbon atoms per chain is given, for example, in the excellent paper by Lewis, Mak and McElhaney [22]. My results agree with these published values to within a degree. An exception are the substransition temperatures. I have measured values similar to the early results [26,30,41,11] which are somewhat lower, however, than the data by Lewis and colleagues [22] or Finegold and Singer [14] *. The experimental section dealing with the pure chainlength effects in this paper is therefore kept at minimum and also no original primary data are shown.

Chainlength effects

The chainlength dependencies of individual transition temperatures T_s, T_p, or T_m are illustrated in Fig. 2. All data pertain to pure 1,2-diacylphosphatidylcholines in excess water. In the case of the pretransition and the chain-melting phase transition temperature the values are reproducible at least to within 0.5 degrees. Quotations for the subtransition temperature are less reliable, only to within ± 5 degrees, owing to the low speed of the $L_\beta \rightarrow L_c$ transformation in multilamellar systems and vice versa. (This is possibly indicative of the kinetic trapping of interlamellar water.) Despite this, the present T_s-results are at least qualitatively representative. On the one hand, the slope of the subtransition temperature versus chainlength dependence is very similar to that observed by Lewis and colleagues [22] (see further discussion), my absolute values being by 5 to 10% lower. On the other hand the subtransition temperature of phosphatidylcholine (triangles) are only by approximately 10% higher than the temperatures at which

* One reason for this discrepancy may be the well known slow kinetics of the formation of two-dimensional lipid crystals. This normally leads to an overestimation of the subtransition temperatures. The other possible source of variability are the differences in vesicle morphology which also affect this kinetics: the number of lipid lamellae in my vesicles was probably relatively small compared to other calorimetric experiments, owing to the special conditions of the sample preparations for this study.

rotator transition sets in for the corresponding chain-length paraffins (vertical tics on the 'error bars' in the lowest curve of Fig. 2).

For the lipids with long hydrocarbon chains the width of the gel-phase region, this is, the relative difference between the subtransition temperature and the chain melting temperature, is nearly constant, approx. 30 K (Fig. 2). Conversely, the width of the region encompassing undulated bilayers becomes relatively insensitive to the lipid chainlength in the range of short chains: for small n_C one has: $(T_m - T_p)_{n_C \to 9} \approx 20$ K.

An upper characteristic temperature limit and a corresponding upper chainlength limit, moreover, define the range of the existence of the periodic, undulated bilayer structures. For phosphatidylcholines *in water* such characteristic temperatures are: $T_{p,max} \approx 70\,^\circ$C and $n_{C_{max,p}} \cong 21$–22, respectively. Outside these boundaries lipid bilayers do not undulate, probably due to the prohibitively strong inter-chain coupling, or else, owing to the fact that lipid headgroups can attain desired conformation without perturbing the apolar chains markedly.

Another set of related data is given in Fig. 3. Increasing sodium chloride concentration from this picture is seen to increase the bilayer pretransition as well as the chain-melting phase transition temperature of various 1,2-diacylphosphatidylcholines. The net effect becomes

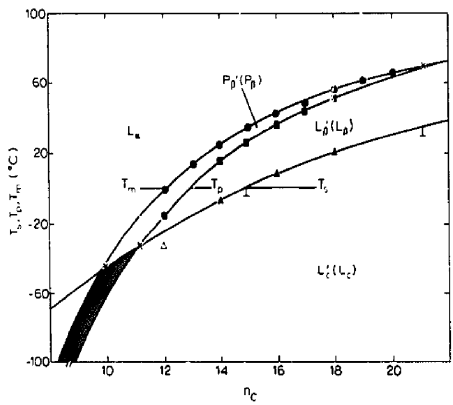

Fig. 2. Partial phase diagram of phosphatidylcholine/water mixtures as a function of the lipid chainlength, n_C. Experimental values of the lipid subtransition (T_s, triangles), pretransition (T_p, squares) and chain-melting transition temperature (T_m, dots) agree nearly quantitatively with the calculated curves. Asterisks give the position of the extrapolated triple points occurring at each edge of the $P_{\beta'}$-phase region. Pretransition temperatures for the odd-numbered phosphatidylcholines stem from Parente and Lentz [27]. Subtransition data can be also found in Refs. 26, 30, 41, 11 and 22. The latter set of data by Lewis et al. is approximately 10–20 degrees (two lengths of the error bars) above the lowest curve throughout. Error bars on the latter curve give the difference between present values of the phosphatidylcholine subtransition temperature and the rotator phase transition temperatures of the even-numbered paraffins.

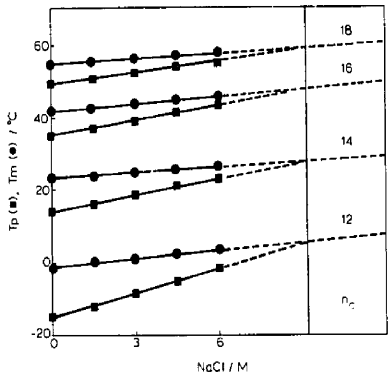

Fig. 3. Pretransition temperature (squares) and chain-melting phase transition temperature (dots) of 1,2-diacylphosphatidylcholines with increasing number of carbon atoms per chain, n_C, as a function of the bulk concentration of sodium chloride. Extrapolated T_p vs. NaCl-concentration lines always cross the corresponding T_m curves at nearly identical extrapolated salt concentration of 10 M, where $T_p = T_{p,max} = T_m$. The difference $T_m - T_p$ tends to zero with increasing chainlength to vanish for $n_C \geq 21$.

less with increasing chainlength, however, the ratio of the T_p vs. [NaCl]- and T_m vs. [NaCl]-slopes being approximately the same, 3.5 ± 0.2, and not too different from the ratio of the corresponding transition enthalpies for the short chain phosphatidylcholines, $(2^{\prime} \cdot 18)/(4.5 \pm 0.8) \leq 5 \pm 4$, as concluded from the pres and previous [27,22] studies. Whereas the pretransitic enthalpy is approximately chainlength independent, the chain-melting enthalpy increases with the number of carbon atoms per chain [20,27,22].

It is noteworthy, that the extrapolated T_p vs. [NaCl] and T_m vs. [NaCl] lines for one lipid intersect at similar critical (nominal) salt concentration which depends on the choice of electrolyte. For many salts, including sodium chloride, the extrapolated, required, bulk salt content is beyond the saturation limit. Notwithstanding this, extrapolating experimental curves is useful. It shows that the (pre)transition and the main-transition temperature at the critical concentration for a given chain type are always the same: approximately 5, 29.5, 47, and 58 °C, in the case of 1,2-dilauroyl- through to 1,2-distearoyl-phosphatidylcholine, respectively.

Headgroup effects

It is not possible to study the dependence of the lipid pretransition for a wide range of headgroup types owing to the fact that most of the diacylphospholipids undergo a direct transition from a lamellar-gel into a fluid-lamellar phase without an intermediate $P_{\beta'}$-phase. Notable exceptions at neutral pH are phosphatidylglycerol and amphiphiles related to this lipid, as well as phosphatidylcholine, and dimethylphosphatidylethanolamine ($PE(CH_3)_2$). But the latter lipid is already an extreme

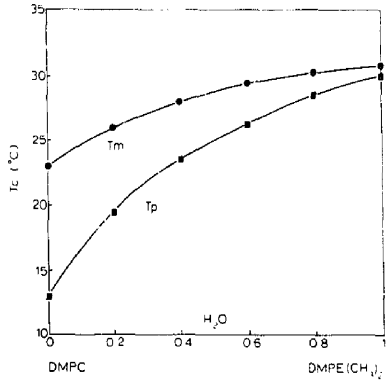

Fig. 4. Pretransition (squares) and chain-melting phase transition (circles) temperature of different mixtures of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-(*N,N*)-dimethyl as a function of the relative concentration of the latter in bilayers in contact with water (heating runs).

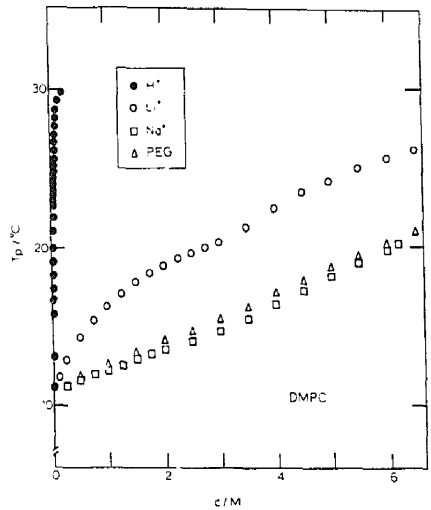

Fig. 5. Effect of the solvent composition on the temperature of the pretransition, T_p, in dimyristoylphosphatidylcholine multilayers. On the scale of the bulk electrolyte concentration, *c*, the pretransition temperature is very sensitive to the choice of the dissolved substance.

case, as indicated by the small width of the P_{β}-phase region for PE $(CH_3)_2$, which in the case of fourteen carbon atoms per chain spans only approx. 1 degree. In order nevertheless to be able to investigate the effect of uncharged polar lipid headgroups on the pretransition temperature I have therefore prepared various mixtures of homologous phosphatidylcholine and dimethylphosphatidylethanolamine. In this way the range between the pretransition temperature of pure phosphatidylcholine in water and the maximal possible pretransition temperature was covered.

Pretransition and chain-melting phase transition temperatures of various mixtures of dimyristoylphosphatidylcholine and corresponding dimethylphosphatidylethanolamine ($n_C = 14$) are shown in Fig. 4. The data indicate that no phase separation occurs for these two lipids: the pretransition as well as the main-transition temperatures of PC and $PE(CH)_2$ follow two smooth curves between 13 and 29 or 23 and 30 °C, respectively, without an indication of additional phase changes. As a function of the changing molar lipid ratio the pretransition-curve, however, has a slope approximately 5-times greater than the chain-melting curve.

Solvent effects

To gauge how significant solvent binding is for the lamellar-to-undulated-gel-phase transition, the pretransition and the main transition temperature of 1,2-dimyristoylphosphatidylcholine were measured as a function of the composition and the concentration of the bathing solution. Binding and non-binding, ionic and non-ionic polar substances were present in the aqueous subphase. The obtained results are widely different on the concentration scale as is shown in Fig. 5. Increasing proton concentration, for example, which leads to the protonation of the phosphate groups, elicits the greatest effects, followed by the lithium ions which also bind to the same groups but with approximately 100-times smaller affinity than protons. (A complete analysis of the lithium binding data to phosphatidylcholine and other phospholipids will be published separately.) Sodium chloride and poly(ethylene glycol) 1000 do not bind to phosphatidylcholine and cause effects which are even smaller, by a factor of approximately 5. The effect of small sugars, which I have already discussed elsewhere

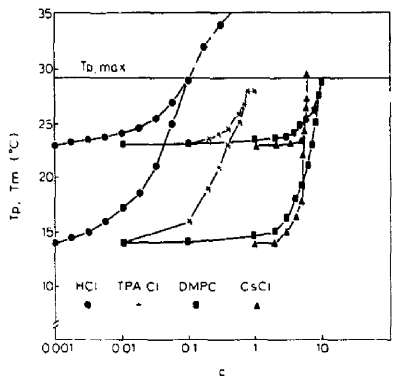

Fig. 6. Pretransition (T_p, lower set of curves) and the chain-melting phase transition temperature (T_m, upper curves) of 1,2-dimyristoylphosphatidylcholine (DMPC) multilayers as a function of the lipid or solute concentration in water, *c* on the logarithmic scale. In harmony with the present theory the limiting value $T_{p,max}(n_C = 14) = 29.5$ °C is insensitive to the origin of the transition temperature shift. (TPA·Cl is tetrapropylammonium chloride.)

[9], is slightly greater and significantly different on the concentration scale for the different carbohydrate types. But on the water-activity scale all investigated sugars affect the pretransition temperature similarly if added to the final liposome suspension. The same is true for non-ionic polymers of different size.

A related set of data including the chain-melting transition temperature and covering a wider range of pretransition temperatures for other substances is presented in Fig. 6. Mean values of the heating and cooling scans are given. From these more complete results and from Fig. 4 it is clear that the pretransition and the main, order-disorder lipid transition for a given chain-length coincide at a point which is quite insensitive to the type of solute; for $n_C = 14$ this point is reached at temperatures close to 30 °C. It is thus identical within experimental error with the extrapolated critical transition temperature (triple-point) for dimyristoylphosphatidylcholine in solutions of sodium chloride (cf. Fig. 3).

Calculation of the transition temperatures

To calculate the critical temperatures of the bilayer subtransition, pretransition, and chain-melting phase transition as a function of the hydrocarbon length and in order to identify the range of the existence of the undulated lipid phases I have elaborated on a model which I have already briefly introduced previously. This is based on a related treatment of the polymorphism of alkanes by Ishinabe [19].

Ishinabe has assumed that inter-chain interaction potential can be expressed as a function of the translational, orientational, and rotational displacements of the chains (ch). If the headgroups (hg) are treated analogously as far as their rotation is concerned, one obtains

$$U = U_{ch} + U_{hg} = A_{ch} \cos m\alpha + B_{ch} P_2(\cos\gamma) + C_{hg} \cos n\alpha$$

$$m, n = 1, 2, \ldots \qquad (1)$$

where γ is the angle between the chain or headgroup axes and α is the sum of the relative angles of rotation of the two neighbouring chains [19]. P_2 is a second order Legendre polynomials and A_{ch}, B_{ch} and C_{hg} are coupling constants.

Based on a simpler version of Eqn. 1, in which the headgroup-dependent term is lacking, and using standard statistical mechanical methods Ishinabe has shown that simple hydrocarbons should undergo a transition into a state of freely rotating chains at a temperature $T_s = A_{ch}/2.5k$, and a transformation into an orientationally disordered, molten phase at $T_m = B_{ch}/k$; k is Boltzmann constant. For lipids I furthermore surmise the existence of an additional (pre)transition into a state of freely rotating heads for the temperature $T_p = C_{hg}/2.5k$.

To bring Eqn. 1 into a practically useful form one can assume that all headgroup- and solvent-dependent end-effects are sufficiently small to justify a perturbative treatment. The variation of the coupling parameters with the hydrocarbon chain length may then be written in the form of a phenomenological expansion

$$X(n_C) = \Delta x(n_C - n_{xC} + n^*_{xC}/n_C)$$

where n_C denotes the number of carbon atoms in each hydrophobic chain. The term n_{xC} corresponds approximately to the length of the shortest segment for which a given phase transition ($x = s, p, m$) is possible and the n^*_{xC} term in the framework of the present approximation allows for the real head-group effects [8]; Δx gives the incremental change per CH_2-group of each coupling parameters, $X = A_{ch}, B_{ch}, C_{hg}$, with $x = a_{ch}, a_{hg}, a_{ch}$. The temperature at which the hydrocarbon chains begin to rotate, i.e., the 'rotator' or subtransition temperature, in this case can be expressed as

$$T_s(n_C) = T_s(\infty)(1 - n_{aC}/n_C + n^*_{aC}/n^2_C) \qquad (2)$$

Similar formal equations can be written for the pretransition and for the chain-melting phase transition temperature as well.

Comparison between Eqn. 2 and the experimental data on phosphatidylcholine subtransition (Fig. 2, squares) yields:

$$T_s(n_C) = 414(1 - 6.25/n_C + 17.5/n^2_C)\ \text{K} \qquad (3)$$

where the parameter $T_s(\infty)$ is identified with the rotational phase transition temperature of the extended-chain crystal of polyethylene, 414 K. If instead of the data given in Fig. 2 the more complete (but slightly non-monotonous) data-set by Lewis et al. [22] is used, optimization yields: $T_s(n_C) = 414(1 - 6.26/n_C + 25/n^2_C)$. Both expressions are numerically similar to the corresponding expression for the rotator phase transition of the crystalline simple hydrocarbons, $T_{s,alkane}(n_C) = 414(1 - 6.5/n_C + 19.4/n^2_C)$ K.

Chainlength dependence of the gel-to-fluid phase transition temperature for the diacylphosphatidylcholines (Fig. 2, circles), which at the molecular level is believed to be well understood ([23,25], see also Ref. 8 and references therein), is well described by the following phenomenological expression:

$$T_m(n_C) = 400(1 - 1.8/n_C - 25.2/n^2_C)\ \text{K} \qquad (4)$$

where the limiting transition temperature now corresponds to the chain-fluidization temperature of the hydrocarbons with infinitely long chains, $T_m(n_C = \infty) = 400$ K.

If the experimentally established chainlength dependence of the lamellar-to-periodic-gel phase transition, this is, of the pretransition temperature of diacylphosphatidylcholines (Fig. 2, triangles), is analyzed from Eqn. 2 one gets:

$$T_p(n_C) = 414(1 - 2.8/n_C - 21.5/n_C^2)\ \mathrm{K} \quad (5)$$

The last term is now negative, probably owing to the negative contribution from the interfacial free energy change at the corresponding phase transition. Slightly different combinations of precise parameter values ensure comparably good agreement with experimental data (for example: $n_{aC} = 2.5$ and $n^*_{aC} = -25.2$) but qualitative conclusions are always the same.

The disparity of the chainlength dependence of the subtransition, the chain-melting, and the pretransition temperatures causes the curves along which the experimental T_s, T_p, and T_m data-points are located to intersect. This gives rise to three triple points (full stars in Fig. 2) which involve the undulated bilayer phase. For lipids with relatively short apolar part the P_β or $P_{\beta'}$ phase, therefore, is unlikely to exist. The critical number of carbon atoms per acyl chain for this is found, dependent on the precise subtransition temperature values used, to be approximately ten to twelve. When the undulated bilayer phase does form, the width of the corresponding region decreases with increasing chainlength until an upper triple point in water (star) is reached for $n_C \approx 21$–22.

The chain-length dependence of the limiting pretransition temperature from the data of Figs. 3 and 6 is found to be

$$T_{p,lim}(n_C) = 414(1 - 2.8/n_C - 14.5/n_C^2)\ \mathrm{K} \quad (6)$$

This indicates that the transitional change of the interfacial contribution (n^*_{aC}) upon approaching the triple point between phases L_β, P_β, L_α becomes less negative. For example, if the contribution from the interfacial region becomes smaller and the term $n^*_{a_{ch}C}$ gets less, the pretransition shifts upward close to the chain-melting phase transition temperature (see further discussion). Stronger interfacial effects (relatively graet $n^*_{a_{ch}C}$) have the reverse effect. They may, ultimately, cause the pretransition to coalesce with the subtransition and cause a lamellar gel-phase to disapepar. The former case is illustrated, for example, in Fig. 4; the latter situation is found for phosphatidylcholines in the presence of certain interfacially active polar substances (to be published).

Molecular origin of the ripple formation

The solvent or the headgroup dependence of the pretransition temperature can not be analyzed quantitatively within the framework of the perturbation theory sketched in the previous section. All that one can do on the basis of such models is to argue that the headgroup–headgroup and the headgroup–solution interactions are involved in the generation of the bilayer ripples. In order to elucidate the precise origin and character of the bilayer pretransition it is, therefore, necessary to investigate the intermolecular interactions in more detail. This has been attempted previously [21,12,3,13,29,24,2,16,35] but none of the available models is as yet capable of predicting *all* relevant experimental facts.

It is rather sure that the bilayer ripples are not a consequence of the hydrocarbon chain tilt, as is often erroneously believed. Many lipids form highly tilted, completely flat bilayers even when they do not contain much water. Phosphatidic acid, phosphatidylserine, and, probably, phosphatidylethanolamine at low pH, for example, form poorly hydrated planar lamellar systems with such tilted chains [6]; chains in one type of the anhydrous phosphatidylethanolamine crystals are also tilted by nearly 40 degrees [34]. And yet, in none of these systems undulations are observed! But bilayer rippling is also not merely a result of the water-mediated coupling between the adjacent lipid membranes. Such conclusion is vindicated by the fact that giant unilamellar vesicles can undergo a pretransition and are rippled [27]. In the following I therefore analyze the changes in the intermolecular coupling at both ends of the phospholipid molecules, which, I believe, are involved in the formation of the membrane ripples, the driving force being the interfacial tendency for a lateral expansion (the head-water-head repulsion).

The first structural consequence of the bilayer rippling is sliding of the hydrocarbon chains along each other [35]. From the energetic point of view this must be accompanied by a lowering of the interchain attraction energy. Energy cost of the reduced chain contacts, ΔU_{att}, then should be proportional to the number of methylene (and methyl) groups, Δn_C, which have lost contact,

$$\Delta U_{att}(n_C) = \Delta n_C \Delta u_{att} \quad (7)$$

Another consequence of the bilayer-surface undulation is an interfacial expansion [28] which is also accompanied by an additional water uptake by the lipid headgroups. The transition into a P_β phase therefore is accompanied by a lowering of the bilayer hydration free energy, by an amount $\Delta G_h < 0$, say. Such increase in the negative free energy of the bilayer hydration is the source of work required for decreasing the interchain interactions at the acyl-chain termini. Hydrocarbon sliding and increased bilayer hydration thus can be inferred to be mutually dependent and proportional to the interfacial area increase at the pretransition. The inability of densely packed unsaturated chains to glide along each other freely may be the reason why even very pure

unsaturated phospholipids do not form undulated bilayers

For the sake of simplicity I assume that the translational chain-shift increases linearly with the membrane hydration * and also with the effective lipid affinity for water. To describe the latter it is useful to introduce an interfacial hydrophilicity parameter, σ_p, which is proportional to the surface polarity. This parameter in the first approximation corresponds to the surface density of the local atomic excess charges on the lipid polar residues but may also contain dipolar and multipolar contributions [4]. The condition for the existence of the undulated bilayer then can be expressed as

$$\Delta U_{att}(n_C) = \Delta G_h(\Delta n_C, \sigma_p, d_w) \cong \Delta G_h(A_{interface}, \sigma_p, d_w) \quad (8)$$

as a function of the average water layer thickness between the adjacent bilayers d_w. In the linear approximation the bilayer hydration free energy is proportional to the square of the surface local excess charge density [4] and changes, in the first approximation, as a hyperbolic tangent with the interfacial separation:

$$G_h(A_{interface}, \sigma_p, d_w) = N_A A_{interface} \frac{\sigma_p^2 \xi}{\varepsilon_0 \varepsilon_\infty} \left(1 - \frac{\varepsilon_\infty}{\varepsilon}\right) \tanh(d_w/2\xi) \quad (9)$$

where $0.07\ \text{nm} \leqslant \xi \leqslant 0.3\ \text{nm}$ is an effective water-order correlation length and ε_0 and ε_∞ are the static and high frequency dielectric constants of water, respectively.

An interfacial area increase, in principle, could be achieved by a uniform tilt of all hydrocarbon chains in one direction. I believe that the reason why this does not happen is the molecular shape-anisotropy. This, together with the bilayer surface limitations on a vesicle surface, energetically favours the formation of ripples. (This may be one of the causes why the pretransition enthalpy diminishes with a vesicle size [27].) A related possibility would be the effects of water-mediated nearest-neighbour head–head interactions (see also Ref 35).

The amplitude, $u_r(n_C)$, and period, $\lambda_r(n_C)$, of the sawtooth-like ripples on the bilayers at least, are in simple geometrical relation **. Therefore, Eqns. 7 and 8 can be used to obtain the following general expression [8] for the modulation wave-vector Q_r of the bilayer ripples

$$Q_r(n_C, d_w) \equiv (2\pi/\lambda_r) = \Delta G_h(A_{interface}, d_w) d_{CH_2} / 2 d_{CC} \Delta u_{att} u_r(n_C) \quad (10)$$

where d_{CC} is the interchain repeat distance and d_{CH_2} is the length of a single CH_2-group.

Eqn. 10 suggests, in accord with experiments, that the modulation wave-vector of the bilayer ripples should decrease upon partial interfacial dehydration until for too-little hydrated bilayers the undulated phase should cease to exist. Upon increasing hydration the reverse effect is predicted by the calculation to occur, in harmony with the observations [40]. In the extreme case bilayers may even interdigitate (to be published).

A more explicit form of Eqn. 10 is found if our previous result for the water dependence of the bilayer free energy of hydration, Eqn. 9, is used. One gets: $Q_r = (d_{CH_2}/2 d_{CC} \Delta u_{att} u_r)\ \Delta G_h(\sigma_p,\ d_w = \infty,\ A_{interface}) \tanh(d_w/2\xi)$. Hydration dependence of any other structural bilayer parameter, such as of the molecular area, or of the bilayer thickness, can be estimated in a similar manner [5].

An approximate value for the incremental change is obtained from Salem's theory [31]. With the published values $d_{CC} = 0.42$ nm, $d_{CH_2} = 0.127$ nm [38,30], and using $G_h(d_w = \infty, A) \leqslant 10\ \text{kJ mol}^{-1}$ one gets $\Delta u_{att} \cong 3.9\ \text{kJ (mol CH}_2)^{-1}$. This together gives for the length of the modulation wavevector

$$Q_r(n_C, d_w) = [0.031/u_r(n_C)] \Delta G_h(d_w = \infty, A_{interface}) \times \tanh(d_w/2\xi) \quad (11)$$

Eqn. 11 can account semi-quantitatively for the recently measured variation with the interfacial separation, and thus with the degree of bilayer hydration, of the reduced modulation wave-vector $\tilde{Q}_r = Q_r \tilde{d}_l$. The latter is equal to the ordinary wave-vector normalized with regard to the apparent lipid layer thickness, $\tilde{d}_l$ [40]. If the modulation wave-vector and the ripple amplitude are assumed to be approximately equal and proportional to the chainlength the agreement between the theory and the experiment is nearly quantitative for an effective water-structure correlation length of 0.3 nm. (Detailed comparison (Fig. 7) is possible on the basis of our previously derived [4] expression $\tilde{d}_l(d_w) = 2 n_C d_{CH_2}(1 - G_{hyd}(d_w)/A_l K_A + d_{head}) \approx 2 n_C d_{CH_2} - 0.3 d_w + 0.6$ nm, where $K_A = 210 \pm 90\ \text{mN m}^{-1}$ is the membrane elastic constant and $d_{head} \approx 0.6$ nm is the thickness of the headgroup region.)

* Owing to the fact that short-range interactions between the lipid headgroups are mainly sensitive to the headgroup and interfacial hydration, this is tantamount to saying that work required for shifting and tilting the chains comes from an effective increase in the interfacial tendency for curvature.

** If it was not for the chainlength dependence of the packing and if no constraints would exist for the bilayer thickness any combination of these two variables with identical ratio would be equally possible and probable. But geometrical system constraints cause the actual values of the structural ripple-parameters to increase approximately linearly with the hydrocarbon chainlength. For phosphatidylcholine the ratio between the undulation period and the lipid layer thickness, for example, attains an approxiamtely constant value close to 2.

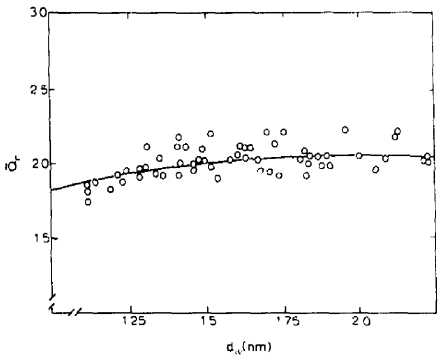

Fig. 7. Functional dependence of the reduced modulation wave-vector of the bilayer ripples, $\tilde{Q}_r \equiv Q_r \tilde{d}_h$, on the interfacial water layer thickness, d_w. Experimental data for $\tilde{Q}_r(d_w)$ (symbols) stem from Ref. 40; theoretical values were calculated by using Eqn. 11 (curve) with parameter values as given in the text. The functional dependence $\tilde{Q}_r(d_w)$ is reminiscent of the spatial tanh-variation of the bilayer free energy of hydration.

A decrease in the bilayer hydration free energy can either be achieved by lowering the actual water content in the system or by osmotically stressing and thus dehydrating lipids [9] until, for approximately twelve water molecules per lipid the minimum required for paying the work of chain displacement is reached. This is the reason why the thermodynamic lipid properties, especially the low-enthalpy transitions, can serve as a basis for a molecular osmometer. Such osmometer can be read-off in absolute units provided that the calibration experiments have been performed. Cooling lipid bilayers below the pretransition temperature, lowering the lipid polarity by modifying the chemical structure of the headgroups, or letting ions, such as lithium or protons, to bind to these headgroups all cause similar effects [10]. The reason for this is that the mentioned, and many other, system changes lower the lipid affinity for water which in the present model is reflected in a reduced value of the hydrophilicity parameter σ_p [4].

Because of the interdependence between the bilayer hydration free energy and the lipid chain melting phase transition temperature [8,10] the criterion for the existence of the lipid pretransition can also be expressed in temperature units. For the lipids with two myristic chains, for example, the pretransition is concluded to vanish when the main transition temperature in excess water becomes greater than 29.5 ± 1.5°C (cf. Fig. 4). The corresponding values for the 1,2-dilauroyl-, 1,2-dipalmitoyl- or 1,2-distearoyl-phospholipids are deduced to be approximately 5, 47, and 58°C, respectively, coinciding with the triple points from Fig. 3. The corresponding expansion series for the limiting pretransition temperature, Eqn. 6, permits other critical transition temperatures to be evaluated easily.

This argument can be extended further to obtain a general scaling-law for the bilayer pretransition temperature. If the temperature-width of the P_h-phase region is plotted as a function of the shift of the chain-melting phase transition temperature (ΔT_m), independent of the choice of the reference state for the calculation or the origin of this shift, the data-points fall all essentially on the same curve (Fig. 8). (The slope of this curve depends slightly on the hydrocarbon chainlength. It decreases moderately with the parameter n_C, its precise magnitude depending on the choice of the reference state.)

In contrast to the enthalpy of the bilayer chain-melting phase transition, the pretransition enthalpy is relatively insensitive to the hydrocarbon chainlength. It decreases, however, dramatically with the number of the lamellae, and perhaps with the size of the vesicles [27]. This indicates that the changes in the interbilayer coupling or in the boundary conditions may also be important for the thermodynamics of the lipid pretransition, as has been speculated earlier [3,16]. But intermembrane interactions are not a conditio sine qua non for bilayer undulation. In my opinion, the real prerequisite for the formation of rippled bilayers is rather the hydration mediated repulsion between the lipid heads and the concomitant increase of the segmental molecular mobility in the interfacial region. Hereby the

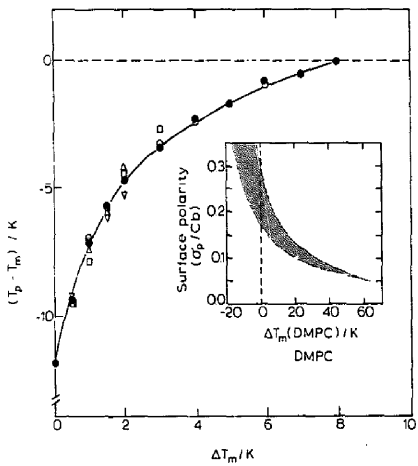

Fig. 8. Width of the P_β-phase region, $T_m - T_p$, determined from Figs. 4, 5, and 6 as a function of the total hydration- or solvent-induced chain-melting phase transition shift, ΔT_m. The latter shift is proportional to the effective surface polarity in excess water and can be expressed in terms of the surface density of the local excess charge, σ_p (see Ref. 10 and Inset). All experimental data points (here for protons (dots), lithium (circles) or sodium (squares) ions, saccharose (triangle down), poly(ethylene glycol) 1000 (triangle up) or decreasing water content (curve)) in such a representation fall on a single line suggesting a simple scaling-law for the pretransition temperature, the interfacial hydration, and the hydration-induced shift or the interfacial solute concentration.

local anisotropy of the head–head interactions as well as the coupling between the heads, the water molecules, and the solutes in the interfacial region all play an important role. The fact that only a few hydration layers close to the membrane surface contribute a lions part to the total hydration free energy of the bilayer-solution system, and thus can drive an interfacial expansion, substantiates this [4,10].

It is noteworthy, however, that the $\Delta T_m = 0$ point in Fig. 8 is arbitrarily chosen so as to correspond to 1,2-dimyristoylphosphatidylcholine in water. But in principle the transition temperatures below 23°C and thus negative transition temperature shifts are possible. Based on the present discussion I infer that the lowest achievable pretransition temperature is likely to coincide with the subtransition temperature. In the case of $n_C = 14$ this implies: $T_{p,lim} = t_s \approx -7°C$. By all means the pretransition of 1,2-dimyristoylphosphatidylcholine can be lowered below 13°C by increasing the lipid headgroup hydrophilicity as is implied in the insert to Fig. 8. Experimental evidence for this will be published later.

Data presented in this report suggest that the shift of the pretransition temperature much more than the chain-melting phase transition shift is determined by the state of the water molecules and by the concentration of the solutes in the *inner part of the interfacial region.* This is owing to the fact that the pretransition is driven mainly by the water-mediated, relatively short-ranged head-head repulsion, whilst the thermodynamic changes at the chain-melting phase transition to a larger extent involve the entire interlamellar aqueous subphase. Pretransition studies, therefore, provide a convenient means for investigating the physico-chemical properties of the region close to the membrane surface, the pretransition temperature acting as a display of an interfacial osmometer (Fig. 9).

The phase boundaries of the symmetric-chain phosphatidylcholines in water, as defined by our phenomenological expressions Eqns. 3, 4, and 5, are shown in Fig. 2. They provide a basis for a rational search for undulated lipid phases.

The same boundaries also contain informations about the triple points for the phases L_β ($L_{\beta'}$), P_β ($P_{\beta'}$), and L_α. At the lower end one has: $T_{p,min1} \approx -45°C$ or $n_{Cmin1,p} \approx 10$ and $T_{p,min2} \approx -35°C$ or $n_{Cmin2,p} \approx 11$, respectively. Below these points the P_β or $P_{\beta'}$ phase is inaccessible (Fig. 2, dark shadow). (The possibility that one such critical point might exist has been discussed previously by Finegold and Singer [14].)

In summary, I have discussed the chainlength variation of the bilayer subtransition, pretransition, and the chain-melting phase transition. I have shown that in the phase diagram of hydrated lipids at least three triple points exist for the rippled (periodic-gel) lipid phase. Undulated lipid bilayers were hypothesized to form

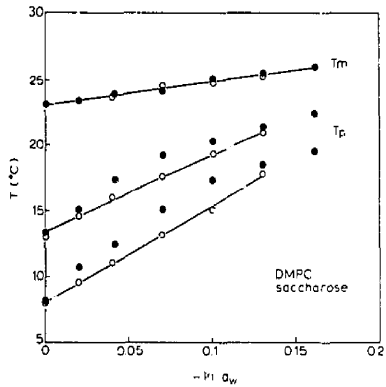

Fig. 9. The importance of solute accessibility for the solution-induced phase transition shift. Saccharose in a suspension of dimyristoylphosphatidylcholine vesicles always causes the bilayer chain-melting phase transition temperature (T_m) as well as lipid pretransition (T_p) to increase, owing to the membrane dehydration. The phase transition temperature shift changes approximately linearly with the logarithm of the water activity coefficient when sugar does not have a direct access to the bilayer surface (open symbols, linear regression lines). In the case that the carbohydrate molecules are present in the interlamellar space the effect is somewhat greater for the pretransition but less noticeably so for the main bilayer phase transition (full symbols). This may be an indication of the relatively high interfacial sensitivity of the pretransition shift.

only when the hydrocarbon chains are of intermediate length and if the lipid heads are sufficiently polar. The reason for this – and for the lipid pretransition in general – is likely to be an intrinsic tendency of the interfacial region to expand upon imbibing water while the overall lipid bilayer is still in the ordered phase. This tendency is relatively unimportant for the lipids with quite short chains, which melt easily, and for the lipids which have long chains and, consequently, are little subject to the chain-end perturbations. The pretransition for diacylphospholipids in water is thus absent if the chains contain less than approx. 10 to 11 and more than 21 or 22 carbon atoms.

All experimentally established facts about the $L_{\beta'} \rightarrow P_{\beta'}$ phase transformation can be understood within the framework of a simple phenomenological theory based on the assumption that the driving force for the bilayer undulation is the interfacial tendency to swell and become more disordered under given conditions. The pretransition temperature is therefore mainly sensitive to the changes in the interfacial region. Within the framework of the present model it is possible to explain nearly quantitatively the variation with hydration of the modulation wave-vector in the rippled phase. Present theory, moreover, provides a rationale for scaling the bilayer pretransition and the chain-melting phase phase transition temperatures to shifts, so that characteristic lipid parameters can be compared directly, independently of their origin. The model introduced in this

work thus not only highlights the molecular mechanism of the bilayer undulation but, moreover, can be used to make specific predictions about the lamellar-to-periodic gel transition and to search for the new regions of the existence of undulated lipid bilayers.

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