Eur Biophys J (1989) 16:369–374

European Biophysics Journal

Can hydration forces induce lateral phase separations in lamellar phases?

G. Bryant and J. Wolfe

School of Physics, The University of New South Wales, Kensington, Australia

Received July 19, 1988/Accepted in revised form November 11, 1988

Abstract. Large repulsive forces measured between membranes of lamellar lipid phases at low hydration are attributed to hydration interactions which vary widely among lipid species. We include this interaction in a model of lamellar phases of two membrane components (two lipids or lipid and protein). The surface polarization of a mixture is taken as a linear combination of those of the components. The model predicts phase separation at low hydration. This may have important consequences for living cells which are dehydrated either by the osmotic effects of tissue freezing, or by desiccation in unsaturated atmospheres.

Key words: Lamellar phase, phase separation, hydration forces, membrane dehydration

Introduction

When lamellar lipid-water systems are dehydrated until the intermembrane separation is a few nanometers or less, very large repulsive forces are measured. These decay exponentially with separation and are attributed to hydration (Lis et al. 1982). The magnitude of the repulsive force is very different for different lipid species (Marra and Israelachvili 1985). In this paper we present an analysis that predicts that this inter-membrane hydration interaction in fluid lamellar phases of mixtures of lipids, or of lipid-protein mixtures, can produce phase separations at low hydration.

Lateral separations of two different, fluid lamellar phases in lipid-water systems have been observed at high hydration or excess water under a number of different conditions (Wu and McConnell 1975; Rand et al. 1980; Rowe 1987; Tamura-Lis et al. 1985). These separations are therefore a consequence of molecular interactions in the plane of the membrane, and can be described in terms of the enthalpy of mixing of the species present. Lateral phase separations have however been observed in biological membranes at low hydration (Gordon-Kamm and Steponkus 1984), and it has been suggested that the lateral stresses produced by the strong hydration repulsion under such conditions can lead to demixing in multicomponent systems (Wolfe 1987). To our knowledge, however, there have been no studies of the phase behaviour of lipid mixtures at the low hydrations where inter-membrane rather than intra-membrane interactions may lead to phase separation.

Very low hydration is associated with damage to biological membranes in both cryobiology and anhydrobiology. When cells are cooled to sub-zero temperatures and then re-warmed, the survival rate can be very low. For some cells, a major cause of the loss of cell viability during a cycle of freezing and thawing is membrane damage due to freeze-induced dehydration. As the temperature is lowered below zero, the solution outside the cell freezes, causing osmotic dehydration of the cell. As the cell shrinks its various membranes are brought into close proximity, and the membrane material forms a multilamellar phase with low hydration. This process leads to severe dehydration at temperatures only a few degrees below zero (Gordon-Kamm and Steponkus 1984). For temperatures T below the freezing point of water T_c, the suction $(-P)$ and the osmotic pressure Π are related by $\Pi - P = 1.22\ (T_c - T)$ MPa K^{-1}.

Studies of the differential behaviour of protoplasts extracted from the leaves of cold acclimated (ACC)* and non-acclimated (NA) rye seedlings during freezing have shown remarkable differences in survival rates, and these survival rates have been correlated with morphological changes that occur in the membranes

* *Abbreviations used*: ACC, cold acclimated protoplasts; NA, non cold acclimated protoplasts; DLPC, dilauralphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; L_α, fluid lamellar phase; H_{II}, inverse hexagonal phase

of both ACC and NA protoplasts in the dehydrated state (Gordon-Kamm and Steponkus 1984). They found that when suspensions of protoplasts were cooled to −10 °C, the membranes of NA protoplasts exhibited separation into particle-rich phases (interpreted as protein-rich) and aparticulate lamellae. In the aparticulate regions, areas of lamellar (L_α) and inverse hexagonal (H_{ii}) phases were observed. However, ACC protoplasts cooled to −10 °C exhibited only small areas of lateral phase separation and no H_{ii} formation. The differences in lipid composition between the plasma membranes of ACC and NA protoplasts are now known (Lynch and Steponkus 1987), but the basis for their different phase behaviour remains obscure. The morphological changes in membranes of both types can be reproduced when the protoplasts are suspended in 5.37 osmolal sorbitol at 0 °C, which produces a similar level of dehydration to cooling to −10 °C. Thus the phase separations that are observed at sub-zero temperatures in NA protoplasts are a result of dehydration rather than the direct effects of temperature.

Even more severe dehydrations are induced by drying biological tissues in air, and seeds and desiccation-tolerant organisms are the most studied systems (Leopold 1986). Equilibration at relative humidities below 90% requires that the chemical potential per unit volume of the remaining water be tens of MPa less than that of the standard state, and so osmotic pressures and suctions of this order are inferred. Large stresses and strains are associated with such dehydrations (Parsegian et al. 1986; Wolfe et al. 1986).

Hydration forces

Studies of the forces between lipid bilayers show that, for water concentrations less than about 70% by weight, the so-called hydration force dominates other inter-bilayer interactions. When two hydrophilic surfaces are brought together, the water between the layers must be forced out. The force per unit area [1] becomes very large at small separations: at 1 nm the repulsion may be as great as 12 MPa. Lis et al. (1982) have conducted osmotic stress measurements on a number of lipid systems, and have characterised the results by fitting an empirical exponential force law for water separations 0.5–3.0 nm (LeNeveu et al. 1976). Over this range, the data ($\mathscr{P}$, y) are well fitted by the equation:

$$\mathscr{P} = \mathscr{P}_0 \, e^{-y/\lambda} , \qquad (1)$$

where $\mathscr{P}$ is the repulsive force per unit area, $\mathscr{P}_0$ is the extrapolated force per unit area at bilayer contact, y is the separation between membranes (strictly the volume-weighted thickness of water and sometimes called d_w) and λ is the characteristic decay length of the force. For pure lipid systems these authors found that the decay lengths were in the range 0.2–0.3 nm for all systems studied. The pre-exponential factor $\mathscr{P}_0$ ranged from $\sim 4 \times 10^8$ to 4×10^9 Nm^{-2} for pure lipid systems, and had an even larger range for mixtures with cholesterol. We propose that this difference in the forces may lead to phase separations in mixed systems, even in the absence of enthalpy of mixing. Figure 1 gives a representation of such a phase separation: a phase which is rich in the more highly repelling lipid species is in equilibrium with another phase, with smaller separation, which has few molecules of the highly repelling species.

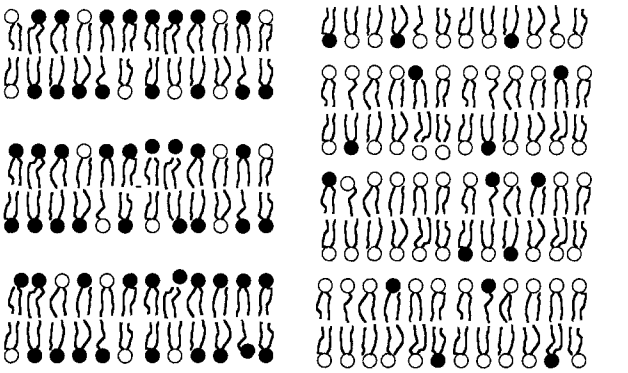

Fig. 1. Sketch illustrating two lamellar phases in equilibrium. That on the left has a larger separation and a higher concentration of the more highly repelling lipid species (shaded in the sketch)

Although the exponential force law used by the authors cited above is empirical, there is some basis in theory. The analysis by Marčelja and co-workers assumes that the lipid surface polarizes the water adjacent to it. The degree of polarization (and thus $\mathscr{P}_0$) is determined by the polarizing properties of the surface, while the decay of the perturbation depends on the properties of the medium (usually water). That is, water molecules adjacent to the surface are oriented by surface dipoles and by discrete surface charges on the polar molecules, thus disrupting the hydrogen bonding network close to the surface. Statistical mechanical models as well as numerical simulations produce an exponential form for the hydration force with decay lengths and $\mathscr{P}_0$ of the correct order of magnitude (Gruen and Marčelja 1983; Kjellander and Marčelja 1985; Attard and Batchelor 1988).

If surface polarization be independent of separation, then the predicted decay length λ is independent

1 Note that in a dehydrated lamellar phase at atmospheric pressure, a repulsion $\mathscr{P}$ between the lamellae must be balanced by the pressure in the intervening water layer which is therefore $-\mathscr{P}$, i.e. it is a suction. Cavitation is however unlikely because the water thickness is less than the critical bubble size

of the lipid species present. (In practice this is only true for incompressible bilayers.) This assumption will be used in the analysis. We note however that a single exponential decay provides an accurate fit to the hydration force data of a particular lipid species only over a limited range of hydration. Over the range considered here, the simple empirical relation allows the hydration force to be included in calculations of the chemical energy of the system.

Theory

In this paper the following assumptions are made:

(1) The lipids are a solution in two dimensions, with the hydration force exerted by the adjacent bilayer treated as an external force,
(2) The solution is ideal, i.e. the enthalpy of mixing is negligible in comparison with the other energies considered here,
(3) All other intermembrane forces are negligible in comparison with the hydration force,
(4) The water phase is incompressible, and
(5) The lipid phase is incompressible in the plane of the bilayer.

Consider a system containing a single lipid species and water. The hydration force per unit area between two adjacent bilayers in a lamellar phase of a single lipid species is, from (1):

$$\mathscr{P} = \mathscr{P}_0 \, e^{-y/\lambda} = b_i^2 \, e^{-y/\lambda} , \tag{2}$$

where b_i^2 replaces $\mathscr{P}_0$ for mathematical convenience. From the hydration force per unit area, the total force on one molecule (with area a_i at the interface) due to the opposing monolayer can be written:

$$\mathscr{F} = (a_i b_i) b_i \, e^{-y/\lambda} \tag{3}$$

for a phase of the single lipid denoted by subscript i.

Consider now a system containing two lipid species characterised respectively by a_1 and b_1 and by a_2 and b_2. We assume that the repulsive force per unit area is linearly dependent on the area fraction of each species (f_1 and f_2) times the value of b_i for that species. (In terms of the model of Gruen and Marčelja (1983), this is equivalent to assuming that the mean surface polarization is linearly dependent on the surface polarization due to the components at the interface.) Repulsion of a single molecule of lipid 1 is also proportional to a_1 so:

$$\mathscr{F}_1 = a_1 b_1 (f_1 b_1 + f_2 b_2) \, e^{-y/\lambda} \tag{4}$$

f_1 and f_2 can be expressed in terms of a_1, a_2 and the number fraction X of species 1 and $(1-X)$ of species 2, whence:

$$\mathscr{F}_i = a_i b_i [(b_1 a_1 X + b_2 a_2 (1-X))/(a_1 X + a_2 (1-X))] \cdot e^{-y/\lambda} \tag{5}$$

The expression in square brackets represents the contribution to the force from one side of the bilayer. To simplify the following algebra we define $\alpha = a_2/a_1$, $\beta = b_2/b_1$, and $Y = y/\lambda$. The total force per unit area between two mixed bilayers is just

$$\mathscr{P} = f_1 F_1/a_1 + f_2 F_2/a_2 \tag{6}$$

whence, replacing f_1 and f_2:

$$\mathscr{P} = (b_1 D(X))^2 \, e^{-Y} , \tag{7}$$

where

$$D(X) = (X + \alpha\beta(1-X))/(X + \alpha(1-X)) .$$

Now the potential energy of a molecule of species 1 due to the presence of the opposing mixed monolayer is given by the negative integral of Eq. (5) with respect to the distance between the layers y. The potential energies of the two lipid species are thus found to be:

$$U_1 = a_1 \lambda \mathscr{P}/D(X) \quad \text{and} \quad U_2 = \alpha\beta U_1 \tag{8}$$

Chemical potential

The equilibrium configuration of the system is determined by the chemical potential, which, in a two dimensional solution, is given by:

$$\mu_i = \mu^0 + k\,T \ln X + \pi a_i/2 + U , \tag{9}$$

where μ^0 is the reference chemical potential, $k \ln X$ is the entropy associated with the molecule, and U is the total potential energy of a molecule due to external interactions, which in this case is the energy due to the hydration force. π is the lateral pressure in the membrane, i.e. twice that in each monolayer. The suction in the aqueous phase acts to compress the phase laterally, and is balanced by a compressive stress in the lamellae. The integral of this stress over the thickness of the membrane gives the lateral two dimensional stress (force per unit length) called the lateral pressure π. For mechanical equilibrium the pressure in the water times the water thickness y must be balanced by this lateral pressure. i.e.

$$-\pi = y P = -\mathscr{P} y \tag{10}$$

At equilibrium the chemical potential must be constant throughout the system for each species present, and the pressure must be constant throughout to ensure equilibrium of the water phase.

Phase separation

If a lateral phase separation exist, then two lamellar phases with different water thickneses and compositions must exist between which the chemical potential

of all components are equal. We thus need to consider two separate phases characterised by the following:

	Phase 1	Phase 2
Volume weighted water thickness	y	y'
Local mole fraction of lipid 1	X	X'
Local mole fraction of lipid 2	$1-X$	$1-X'$

(see Fig. 1). The conditions for equilibrium in this system are

$$\mu_1=\mu_1', \mu_2=\mu_2' \text{ and } \mathscr{P}=\mathscr{P}', \tag{11}$$

where the third equation comes from the equilibrium of the water phase, i.e. $P=P'$.

Thus, using Eqs. (7)–(11), a solution can be found where two lamellar phases coexist. After some algebra these equations reduce to the following simultaneous equations:

$$\alpha \ln(X/X')\,[\ln(D/D')+\beta/D-\beta/D'] = \ln\{(1-X)/(1-X')\}\,[\ln(D/D')+1/D-1/D']$$
$$\mathscr{P} a_1\lambda/kT=-\ln(X/X')/[\ln(D/D')+1/D-1/D'],$$
$$\text{where } D=D(X),\ D'=D(X'), \tag{12}$$

These equations are not analytically soluble, and because of the nature of the logarithmic functions involved some difficulties with precision are encountered when using numerical techniques. At least 20 digits of precision and some judicious choices for initial values are required to find solutions over the range presented.

Results and discussion

The results presented here are for lipid systems at 298 K with the more repelling lipid having the following parameters: $a_2=.687\ \text{nm}^2$, $\lambda=\lambda_2=.26$ nm, $b_2^2=525$ MPa (Data for DLPC from Lis et. al. (1982)). The other parameters are α, the ratio of areas of the two lipids, and β, where β^2 is the ratio of the preexponential factors. Figure 2 shows a plot of the hydrostatic pressure (always negative) in the water phases vs. X, the mole fraction of lipid 1 (or $(1-X)$ the mole fraction of lipid 2). The suctions on the abscissa may appear large, but note that a suction of 10 MPa is produced in unfrozen water by a temperature of $-8.2\,^\circ$C, or in water at 20 °C equilibrated with an atmosphere of relative humidity of 93%. The parameters used are $\alpha=1$ and $\beta=10$ and 5. For lipid systems the areas per molecule are always of the same order and α will usually be in the range $0.5<\alpha<2$. For the values of parameters which we cite here, there is only one solution to Eq. (12); and all configurations corresponding to that solution have lower free energies than, and are thus stable with respect to, the homogeneous phase with the same total composition. (A complete discussion of the stability will be given elsewhere). The solutions

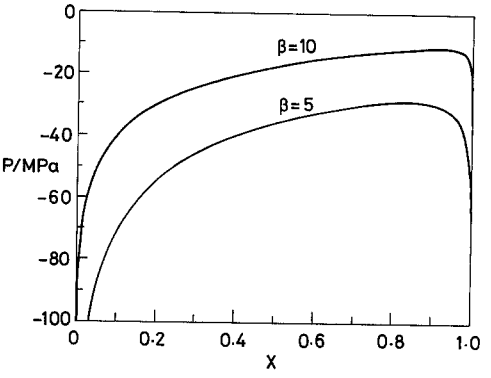

Fig. 2. The figure shows the phase diagram at constant temperature for a binary mixture of lipids for which the enthalpy of mixing is negligible. The abscissa is the number fraction X of the less repelling species (lipid 1). The ordinate is P, the pressure in the aqueous phase between the membranes, which is minus one times the repulsion between membranes. The parameters for the more repelling species (lipid 2) are those for DLPC (data from Lis et al. 1982). α is taken as 1 in both curves, but different repulsions ($\beta=5$ and 10) are shown

have a strong dependence on β. As β is decreased the minimum suction for phase separation increases (i.e. pressures become more negative), and the phase diagram becomes more symmetric.

These results may in principle be applied to a number of lipid-water systems, although numerical predictions are difficult as hydration force data are rather scarce. Lis et al. (1982) present data for several pure lipids (mainly PC), and for lipids with cholesterol. From these data b_i^2 ranges from a few MPa to several thousand MPa. However these large values are for mixtures with cholesterol and are difficult to quantify for individual lipids. Marra and Israelachvili (1985) have shown using direct force measurements on curved mica surfaces that at separations less than ~ 2 nm the hydration repulsion of DPPC is greater than that of DPPE by several orders of magnitude. No quantitative estimates of the hydration force are possible from this technique however, as the force can not be measured accurately at separations of less than ~ 1 nm. Give the range of hydration forces known to exist in the relatively few lipid species whose hydration properties are known, the values of β used in these calculation are not extreme and so it appears likely that phase separations will be found to occur in some mixed lipid systems at low hydration.

The results presented above may also be applied to mixtures of proteins and lipids. The hydration repulsion between proteins are not known to us, but some order of magnitude calculations may still be made. We make the assumptions that the hydration force at a protein's surface is similar to that for a lipid but that the protein spans the bilayer and extends a distance d_p beyond the bilayer. The area of the protein is a_p and is

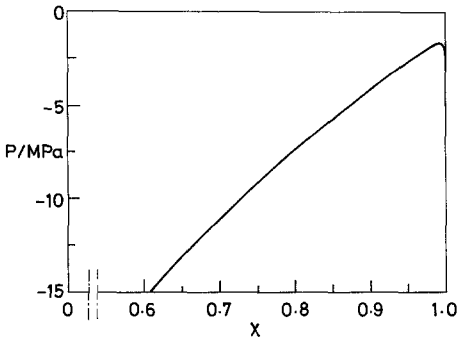

Fig. 3. This figure shows the phase diagram for a model of a mixture of proteins and lipid. Again the lipid is taken to be DLPC, and the parameters which model the protein are $\alpha = 20$, $\beta = 5$ and $d_p \approx 0.4$ nm. The protein is the more strongly repelling component in this case, so $1 - X$ is the number fraction of protein and X the number fraction of lipid. Phase separations occur at much smaller suctions than those in Fig. 1, and the separation forms a nearly pure lipid phase at small separation in equilibrium a mixed protein-lipid phase with a larger separation

considerably larger than the area per lipid. (The size and shape of proteins vary considerably, so any values used will necessarily be approximate.)

Because the protein extends beyond the bilayer, its hydration force becomes large at greater membrane separation. If we assume that the preexponential factor at the protein surface is equal to b_1^2 (i.e. the same as for the lipid), then the hydration repulsion at inter membrane separation y will be given by:

$$b_2 = b_1 \exp(d_p/\lambda). \tag{13}$$

For instance, $\alpha = 20$ and $\beta = 5$ would correspond to a medium size protein (molecular weight $\sim$30,000 Daltons and $d_p \approx .4$ nm). The equations are then identical to those for mixtures of pure lipids, but the ranges of values for α and β are different. Figure 3 represents the phase diagram for DLPC mixed with the above proteins. As can be seen from the diagram, phase separations are predicted at quite modest pressures, forming a nearly pure lipid phase and a mixed protein-lipid phase. That is, the membrane separates into two mixed lipid-protein phases of different composition and repeat spacing[2]. (Note that lipid-protein mixtures will not form lamellar phases if $1 - X$, the mole fraction of protein, is too high. This phase diagram thus only applies at protein concentrations low enough to form a lamellar phase at negative pressures less than $\sim$1 MPa).

Proteins are too varied and complicated for us draw quantitative conclusions from these results. They do however illustrate a mechanism whereby hydration forces give rise to phase separations in protein-lipid systems at low hydration. Gordon-Kamm and Steponkus (1983) have observed such phase separations in freeze fracture studies of NA protoplasts at low hydration produced either by sub zero temperatures or osmotic contraction. These authors noted that further dehydration leads to areas of H_{ii} formation. It is possible that lateral phase separation of lipid species play an important intermediate state in the formation of the inverse hexagonal phase at low hydrations. Protoplast plasma membranes, for example, contain mixtures of strongly hydrating lipids (such as PC) and weakly hydrating lipids (PE and glucoserebrosides). Our model suggests that, when water is removed from the system, phase separation will occur. The weakly hydrating lipids will form relatively pure phases devoid of protein. Those lipids which tend to form the H_{ii} state (such as PE) are thus concentrated in the absence of proteins, and this may facilitate the formation of a hexagonal phase. Whether lateral phase separations in such lipid mixtures exist independently over a range of hydrations, or whether they be simply an intermediary for H_{ii} formation, is a matter for conjecture. In either case, however, lateral phase separations may have a crucial part to play in the morphology of such lipid water systems at low hydration.

Acknowledgements. We thank P.L. Steponkus, whose discussions about membrane morphology suggested this study, and J.R. Smith for comments on the manuscript.

References

Attard P, Batchelor MT (1988) A mechanism for the hydration force demonstrated in a model system. Chem Phys Lett 149:206–211

Gordon-Kamm WJ, Steponkus PL (1984) Lamellar-to-hexagonal$_{II}$ phase transitions in the plasma membrane of isolated protoplasts after freeze-induced dehydration. Proc Natl Acad Sci USA 81:6373–6377

Gruen DWR, Marčelja S (1983) Spatially varying polarization in water. J Chem Soc Faraday Trans 2:225–242

Gruen DWR, Marčelja S, Parsegian VA (1983) Water structure near the membrane surface. In: De Lisi C et al. (eds) Cell surface phenomena. Alan R Liss, New York, pp 59–91

Kjellander R, Marčelja S (1985) Pertubation of hydrogen bonding in water near polar surfaces. Chem Phys Lett 120:393–396

Le Neveu DM, Rand RP, Parsegian VA (1976) Measurement of forces between lecithin bilayers. Nature 259:601–603

Leopold AC (1986) Membranes, metabolism and dry organisms. Cornell University Press, New York

Lis LJ, McAlister M, Fuller N, Rand RP, Parsegian VA (1982) Interactions between neutral phospholipid bilayer membranes. Biophys J 37:657–666

Lynch DV, Steponkus PL (1987) Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L cv Puma). Plant Physiol 83:761–767

Marra J, Israelachvili J (1985) Direct measurements of forces between phosphatidylcholine and phosphatidylethan-

2 We emphasise that this is not the only mechanism which could lead to separation of a protein-rich phase, and an anonymous reviewer has pointed out that variations in membrane thickness could also lead to such a separation

olamine bilayers in aqueous electrolyte solutions. Biochemistry 24:4608–4618
Parsegian A, Rau D, Zimmerberg, J (1986) Structural transitions induced by osmotic stress. In: Leopold AC (Ed) Membranes, metabolism and dry organisms. Cornell University Press, New York, pp 306–317
Rand RP, Parsegian VA, Henry JAC, Lis LJ, Mc Alister M (1980) The effect of cholesterol on measured interaction and compressibility of dipalmitoylphosphatidylcholine bilayers. Can J Biochem 58:959–968
Rowe ES (1987) Induction of lateral phase separations in binary lipid mixtures by alcohol. Biochemistry 26:46–51
Tamura-Lis W, Cunningham BA, Lis LJ, Reber EJ, Collins JM (1985) Ca^{2+} induced phase separations in mixtures of zwitterionic phospholipids Biophys J 47:423a
Wolfe J (1987) Lateral stresses in membranes at low water potential. Aust J Plant Physiol 14:311–318
Wolfe J, Dowgert MF, Maier B, Steponkus PL (1986) Hydration, dehydration, and the stresses and strains in membranes. In: Leopold AC (ed) Membranes, metabolism and dry organisms. Cornell University Press, New York, pp 286–305
Wu SH, Mc Connell HM (1975) Phase separations in phospholipid membranes. Biochemistry 14:847–854