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LIPID PACKING AND TRANSBILAYER ASYMMETRIES OF MIXED LIPID VESICLES

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Summary

Predictions, based on a previously developed theory, of the radii and asymmetric lipid distribution of mixed phosphatidylcholine/lysophosphatidylcholine and phosphatidylcholine/cholesterol vesicles of variable composition are presented. The results compare well with available experimental data, except for *cis*-unsaturated phosphatidylcholine/cholesterol vesicles at high concentrations of cholesterol. It is concluded that specific lipid-lipid interactions need not be invoked for saturated and *trans*-unsaturated phosphatidylcholine mixed with lysophosphatidylcholine or cholesterol. A discussion of the effect of packing stresses on induced flip-flop and non-spherical vesicles is also presented.

Introduction

There are at present two different, though not necessarily incompatible, theoretical approaches to describing the physical mechanisms governing lipid and membrane self assembly. These may be loosely grouped into continuum and molecular theories. The description of a membrane in terms of a two-dimensional elastic sheet [1] or in terms of an elastic lamellar liquid crystal [2] allows for certain macroscopic properties of membranes, such as their overall shape and curvature, to be quantitatively analysed. These approaches are similar in spirit to the 'bilayer couple hypothesis' proposed by Sheetz and Singer [3], which has been given a quantitative theoretical framework by Evans [1]. A different approach is to derive the structure and properties of lipid and lipid-protein assemblies in terms of the molecular packing characteristics of the

constituent molecules [4—6]. In this approach the concepts of the competing effects of interaction energies, entropy, and the geometrical packing constraints of molecules are brought together in one general theory which has successfully predicted many of the physical properties of one component lipid micelles, bilayers and vesicles [5,6] and has been further extended, qualitatively, to biological membranes [7,61].

While continuum theories of membranes are particularly suitable for theoretical interpretations of the macroscopic shapes of cells and other large membranous structures [8], a molecular theory is needed for analysing smaller structures such as micelles, vesicles, membrane pores, etc., and especially for multicomponent systems when highly complex and specific local interactions are present (e.g. specific lipid-lipid and lipid-protein interactions, boundary lipids, local membrane protrusions, clustering, aggregations and phase separations [61]). So far the molecular theory has been applied to simple cases of pure one component vesicles, and the general properties of two-component vesicles. In this paper we apply the theory to a detailed quantitative analysis of mixed (two-component) vesicles made up of lipids of very different packing properties (phosphatidylcholine, lysophosphatidylcholine, and cholesterol). These lipids also appear to have very different physiological functions: thus phosphatidylcholine is one of the main constituents of cell membranes (apart from plant cells); cholesterol is another abundant lipid but is more specifically distributed in membranes that display more rigidity, e.g. myelin; lysophosphatidylcholine is found in cell membranes as 1—4% of total phospholipid. In particular lysophosphatidylcholine is a potent fusogenic and lytic agent and can cause membrane shape changes even in small amounts. It is also more abundant in some secretory cells.

The good quantitative agreement we obtain between theory and experiment of these mixed lipid vesicles gives much insight into why cholesterol and lysophosphatidylcholine affect membranes in the way they do, and, more generally, should give some confidence in further applying the theory to more complicated multicomponent (lipid-protein) systems.

Theory

A unified theory of the self assembly of lipids into micelles, vesicles and bilayers has been presented [5,6]. This theory incorporates the concepts of solution thermodynamics, interaction free energies, entropy of mixing and molecular geometry in a simple way. Since the theory has been well discussed elsewhere we continue with the notation previously introduced and first summarise only the results already obtained for mixed lipid vesicles which are relevant to the present application. Further theoretical results are then derived. A more detailed consideration of the basis and approximations of the theory is relegated to the Appendix.

Initially we consider spherical vesicles formed by, for example, cosonication of two lipids A and B. At equilibrium A_0 and B_0 denote the number of lipids of each species in the outer monolayer, and A_i and B_i are the number in the inner monolayer.

For convenience the lipid distributions are described in terms of the fol-

lowing quantities

$$N = A_0 + A_i + B_0 + B_i$$

$$F = (A_0 + A_i)/N$$

$$f = (A_0 + B_0)/N, \text{ i.e. } f/(1-f) = (A_0 + B_0)/(A_i + B_i) \quad (1)$$

$$X_0 = A_0/(A_0 + B_0)$$

$$X_i = A_i/(A_i + B_i) = (F - X_0 f)/(1-f)$$

where N is the total number of lipids/vesicle and F is the total fraction of lipids A in the vesicle, equivalent to the mol fraction of lipid A in the system. f gives the fraction of lipids in the outer monolayer. For a symmetric lipid distribution the fraction of lipid A in the outer and inner monolayers are both equal to F. That is, for no asymmetry $X_0 = X_i = F$, whereas for asymmetric vesicles X_0 and X_i will deviate from F. in Ref. 6, and more rigorously in the Appendix, it is shown that at 'equilibrium' * all mixed lipid vesicles should be asymmetric. Defining the asymmetry as the difference between X_0 and F the condition for a minimum free energy may be written as

$$\Delta X_0 \equiv X_0 - F = \frac{\gamma(1-F)F}{R_0 kT}\,\delta \quad (2)$$

where

$$\delta = [Fa_A + (1-F)a_B](D_A - D_B) + \left(\frac{R_i}{R_0}\right)^2 [FD_A + (1-F)D_B](a_A/R_A - a_B/R_B)t \quad (3)$$

and further

$$\Delta X_i \equiv X_i - F \approx -\left(\frac{R_0}{R_i}\right)^2 \Delta X_0 \quad (4)$$

Here R_0 and R_i are, respectively, the outer and inner mixed vesicle radii measured at the hydrocarbon-water interfaces, R_A and R_B are the outer radii of the pure vesicles of lipids A and B defined by Eqn. 7, $t = (R_0 - R_i)$ is the hydrocarbon bilayer thickness, a_A and a_B are the optimal surface areas of the lipids A and B measured at the hydrocarbon-water interfaces, D_A and D_B are the effective head group lengths, γ is the hydrocarbon-water interfacial free energy (approx. 50 erg/cm^2 [5]), $\mathbf{k}$ is Boltzmann's constant and T is the temperature.

While Eqns. 2–4 provide a convenient theoretical description of the asymmetry, it appears that the quantity experimentally accessible is the ratio of the number of lipids of one species (A or B) in the outer monolayer to the number in the inner monolayer. These quantities are defined by

$$\frac{A_0}{A_i} = \frac{X_0 f}{X_i(1-f)}$$

$$\frac{B_0}{B_i} = \frac{(1-X_0)f}{(1-X_i)(1-f)} \quad (5)$$

* By 'equilibrium' we do not imply that vesicles are the thermodynamically most favoured state of phospholipid suspensions (cf. p. 1557 of Ref. 5, and p. 200 of Ref. 6).

Unless the asymmetry is large we find that, to a good approximation $f/(1-f) = (A_0 + B_0)/(A_i + B_i) \approx (R_0/R_i)^2$; combining Eqns. 2–5, the equilibrium outside/inside ratio of lipid A is then given by

$$A_0/A_i \approx \left(\frac{R_0}{R_i}\right)^2 \left[\frac{F + \frac{\gamma F(1-F)}{R_0 kT}\delta}{F - \left(\frac{R_0}{R_i}\right)^2 \frac{\gamma F(1-F)}{R_0 kT}\delta}\right] \tag{6}$$

where δ is defined in Eqn. 3. Similarly, B_0/B_i follows from Eqn. 5. When the lipids are symmetrically distributed $X_0 = X_i = F$, and so $A_0/A_i = B_0/B_i = f/(1-f) \approx (R_0/R_i)^2$ as expected from a consideration of the ratio of the outer and inner surface areas.

The equilibrium outer vesicle radius R_0 is governed by geometric constraints imposed by lipid packing, and is given to a good approximation by the geometric 'packing equation' [6]:

$$R_0 \approx \frac{l_c[3 + \sqrt{3(4\bar{v}/\bar{a}l_c - 1)}]}{6[1 - \bar{v}/\bar{a}l_c]} \tag{7}$$

where

$$\bar{a} = X_0 a_A + (1 - X_0)a_B \tag{8}$$

$$\bar{v} = X_0 v_A + (1 - X_0)v_B \tag{9}$$

Here $\bar{a}$ and $\bar{v}$ are the mean outer monolayer lipid surface area and hydrocarbon volume, respectively, v_A and v_B are the hydrocarbon volumes of lipids A and B, l_c is the critical hydrocarbon length (assumed to be the same for both lipids). The inner vesicle radius is then readily obtained by use of volume and area conservation equations (cf. Refs. 5 and 6 and Eqns. A2–A5).

For any given lipid system we can specify a_A, a_B, v_A, v_B, D_A, D_B, and l_c (the large number of variables involved is at first sight daunting but they are all either experimentally accessible or readily estimated (cf. next section)). For any mol fraction F the above equations can be readily solved to obtain all the relevant (i.e. experimentally measurable) quantities.

Finally we mention that the theory indicates that vesicle radii are not discrete but rather have a Gaussian distribution about the mean R_0 value with standard deviation

$$\sigma_R \geq \frac{R_0}{l_c}\sqrt{\frac{kT}{8\pi\gamma}} \tag{10}$$

This is usually a few percent of R_0 but can increase sharply as a consequence of finite head group length [6] and the distribution of asymmetry (see below). The asymmetry is likewise not discrete but is also Gaussian distributed about the mean value of X_0. The standard deviation is

$$\sigma_{X_0} \geq \frac{R_i}{R_0}\sqrt{\frac{F(1-F)}{N}} \tag{11}$$

The $1/\sqrt{N}$ dependence means that the distribution in X_0 is sharply peaked.

Typically, for $N \gtrsim 10^3$ and $F \approx 0.5$ means that $\sigma_{X_0} \gtrsim 3\%$. However the distribution of A_0/A_i values about the mean value will be more broad. The standard deviation is

$$\sigma_{A_0/A_i} \geqslant \frac{(F + R_0^2/R_i^2)}{F^2} \sigma_{X_0} \tag{12}$$

Typically σ_{A_0/A_i} can be 5—10 times larger than σ_{X_0}. Thus we find that the spread in the transbilayer distribution can be fairly large, and is particularly large when N is small (small vesciles) and/or when F is small.

The theory has successfully described phosphatidylcholine/phosphatidylethanolamine mixed vesicles at a single composition: $F = 0.48$ [6]. We shall proceed now to describe phosphatidylcholine/lysophosphatidylcholine and phosphatidylcholine/cholesterol mixed vesicles (in which lipid A will be phosphatidylcholine). Since lysophosphatidylcholine and cholesterol are very different from both phosphatidylcholine and phosphatidylethanolamine these mixed vesicles display a wide variety of observed properties and the present application should then provide a more stringent test of the theory. First, however, we must consider the relevant geometrical variables (a_A, a_B, v_A, l_c, D_A, etc) that characterise phosphatidylcholine, lysophosphatidylcholine and cholesterol. This is the topic of the next section.

Geometrical description of lipid molecules

The results of the previous section are all expressed in terms of quantities that are readily accessible from experiment (interfacial energy γ, hydrocarbon surface areas a, and volumes v, etc.) though there is some disagreement on their exact numerical values. These differences are usually small and do not greatly affect the quantitative theoretical predictions. We consider now the geometrical characterisation of each lipid in turn. The hydrocarbon chain length l_c is discussed separately since it is a sensitive variable.

Phosphatidylcholine (lipid A)

We have previously [5,6] modelled phosphatidylcholine vesicles using measured values of $v_A = 1063$ Å^3, $a_A = 71.7$ Å^2 [9,10] and $l_c \approx 17.5$ Å. The theory then predicts $R_0 \approx 105$ Å, $t \approx 30$ Å, $N \approx 2600$, and $A_0/A_i \approx 2.0$, in good agreement with experiment [11,12]. Note that in our theory R_0, a_A and t refer to the hydrocarbon-water interface and so take values slightly different from those measured since these are usually referred to the ends of the head groups. Thus, the experimental values for R_0 will be greater than the theoretical value of 105 Å by approx. D_A, or a few ångstroms; similarly the measured bilayer thickness t should be larger than 30 Å (cf. experimental values of 37 ± 2 Å and 35 Å [11—14]), and a_A should be larger than 71.7 Å^2 on the outer monolayer and smaller on the inner monolayer (cf. 74 Å^2 and 61 Å^2, respectively, for egg phosphatidylcholine [12], and 76 Å^2 and 68 Å^2 for dipalmitoylphosphatidylcholine [15]). For example, using the above-mentioned experimental results for egg phosphatidylcholine, and taking the length of the polar region to be approx. 4 Å we find the thickness of the hydrocarbon region for egg phos-

phatidylcholine to be approx. 29 Å, and that $a_A \approx 68$ Å^2 and 69 Å^2, respectively, at the outer and inner hydrocarbon-water interfaces, i.e. both areas are about the same, as expected theoretically [5].

The difference in lipid surface areas measured at the inner and outer polar regions is important since some authors [16] have used their measurements of $f/(1-f)$ (i.e. outside/inside lipid distribution) together with independently measured values of the total bilayer thickness (i.e including the head group lengths) to calculate values of R_0. They have further assumed that the area/lipid at the ends of the head groups is the same for both monolayers which, as we have seen, is not the case [12,15]. If, however, we take the head group areas to be at the hydrocarbon-water interfaces, and use $f/(1-f)$ data along with a hydrocarbon region thickness of approx. 29—30 Å, then we find that (i) the inner and outer head group areas are the same, and (ii) the value of $R_0 \approx 120$ Å deduced from some experiments [16] becomes $R_0 \approx 103$—107 Å, which is also in agreement with more direct measurements [11,12]. We adopt this as the 'experimental' value with which we compare our theory.

For the sake of continuity with the earlier work [5,6] we use the values noted above for v_A, a_A and l_c (Table I). It should be borne in mind that differences of a few percent in v_A and a_A do not significantly affect our results.

Lysophosphatidylcholine (lipid B)

By comparison with phosphatidylcholine the geometrical properties of lysophosphatidylcholine have been less extensively studied. The only difference in molecular structure is that lysophosphatidylcholine has one hydrocaron chain whereas phosphatidylcholine has two which suggests that we take a hydrocarbon volume, v_B, for lysophosphatidylcholine equal to half that of phosphatidylcholine ($v_B = \frac{1}{2} v_A$) while the head group area, a_B, and l_c should be essentially the same as for phosphatidylcholine. Thus we use $v_B = 531$ Å^3, $a_B = 71.7$ Å^2 and $l_c = 17.5$ Å for lysophosphatidylcholine, whence $v_B/a_B l_c = 0.42$. The theory predicts (cf. Ref. 5, section 5) that non-spherical micelles should form when v/al_c lies between 0.33 and 0.50. In addition the predicted aggregation number (cf. Fig. 8 of Ref. 5) is approx. $4.4[(4\pi l_c^3)/(3v)] \approx 186$, and we expect that the micelles should be toroidal or dimpled spheroids with an axial ratio of about 2.3. These results compare very well with the experimentally determined [17] aggregation number of 181 for lysophosphatidylcholine micelles and the observation that there was significant deviation from sphericity although the exact shape could not be determined. This good agreement gives us confidence in our geometrical characterisation of lysophosphatidylcholine.

Cholesterol (another lipid B)

In mixed egg phosphatidylcholine/cholesterol monolayers [60] and myelin phosphatidylcholine/cholesterol bilayers [37] cholesterol initially behaves as a lipid of area approx. 19 Å^2 up to a mol fraction of about 0.5. This reflects the condensing effect of cholesterol on phosphatidylcholine. Previous theoretical work [4] on the packing of phosphatidylcholine and cholesterol in mixed vesicles used this hydrophilic surface area (defined at the hydrocarbon-water interface) of 19 Å^2 for cholesterol to accurately fit experimental R_0 vs. com-

position data. It also emerged that the cholesterol molecule behaves as if it has a frayed shape (i.e. a truncated cone) whose hydrophilic head group area (approx. 19 Å^2) is smaller than its hydrophobic end area (approx. 38 Å^2) found when a pure cholesterol monolayer is compressed. The assumption of a frayed shape for cholesterol (as opposed to the tapered shapes of phosphatidylcholine, lysophosphatidylcholine and phosphatidylethanolamine molecules) facilitates the quantitative explanation [4] of the condensing effect of cholesterol in terms of molecular geometry without invoking specific interactions. It also explains why pure cholesterol does not form micelles or bilayers.

We choose the hydrocarbon volume of cholesterol so that the molecule has packing properties consistent with earlier results [4], viz. $v_B \approx 400$ Å^2, $a_B = 19$ Å^2 and $l_c = 17.5$ Å. Other combinations, viz. higher a_B and v_B [4], yield similar results.

Hydrocarbon length, l_c, and head group length D

The theoretical predictions are relatively insensitive to the exact values of v and a but are strongly dependent on the choice of l_c and, to a lesser extent, of D. The maximum value of l_c can be estimated from a knowledge of the hydrocarbon chain length [5] and more closely determined by fitting the measured radii of pure phosphatidylcholine vesicles. The l_c value turn out to be about 80% of the fully extended hydrocarbon chain length [18]. This reflects the large unfavourable entropy associated with hydrocarbon chains adopting non-optimal configurations [19], e.g. by being extended too much.

We take l_c to be in the range 17—17.5 Å for all the lipids. This gives radii for pure phosphatidylcholine vesicles in the range 105—120 Å consistent with the experimentally observed range [7,11,12,20]. The size of pure lysophosphatidylcholine micelles is also successfully predicted, as already noted. Our choice of l_c is also acceptable for cholesterol: Crowfoot [21] quotes 20 Å for the fully extended length of cholesterol and we expect that the terminal chains in the fluid-like bilayer interior to be somewhat less extended.

Though the problem of different chain lengths is clearly important it is theoretically much more difficult to handle and is unlikely to have a large effect in the present application. That lateral phase separations can occur when the hydrocarbon chain lengths differ by more than two carbons [22] further highlights the point.

The effective head group length, D, is the distance above the hydrocarbon-water interface at which the repulsive forces (steric, electrostatic, hydration) act. This distance is expected to be of the order of the polar region or head group thickness. For phosphatidylcholine the head groups appear to lie almost parallel to the surface [23—26] so that D should be less than the fully extended headgroup length (approx. 10 Å) or the distance between the charges of the zwitterionic headgroup (7.7 Å [27]), and will be taken to be about 4 Å for both phosphatidylcholine and lysophosphatidylcholine. For cholesterol, since the position of the molecule in a phosphatidylcholine bilayer is with the terminal hydroxyl group at the hydrocarbon interface [28,29], we take $D = 0$.

This completes the choice of lipid areas, volumes and lengths needed for analysing the properties of mixed lipid vesicles. These are summarized in Table I.

TABLE I

Our choice of hydrocarbon volume v, chain length l_c, head group area a, and effective head group length D for the lipids. For a more detailed discussion see above.

Lipid	v(Å^3)	a(Å^2)	l_c(Å)	D(Å)
Phosphatidylcholine	1063	71.7	17.0—17.5	4
Lysophosphatidylcholine	531	71.7	17.0—17.5	4
Cholesterol	400	19	17.0—17.5	0

Results

We now apply the theory outlined previously to phosphatidylcholine/lysophosphatidylcholine and phosphatidylcholine/cholesterol mixed vesicles, comparing the results, where possible, with experiments. We then discuss the general features of the behaviour of these systems.

Phosphatidylcholine/lysophosphatidylcholine

Using the appropriate values for v, a, l_c and D from Table I, we obtained the results shown in Fig. 1 for the variation of mixed vesicle radius R_0, and transbilayer distributions for both phosphatidylcholine (A_0/A_i) and lysophosphatidylcholine (B_0/B_i), as the fraction of lysophosphatidylcholine in the vesicles is increased. We now compare these calculations with experimental results.

De Kruijff et al. [16] measured the inside/outside ratio of total phospholipid of cosonicated phosphatidylcholine/lysophosphatidylcholine vesicles con-

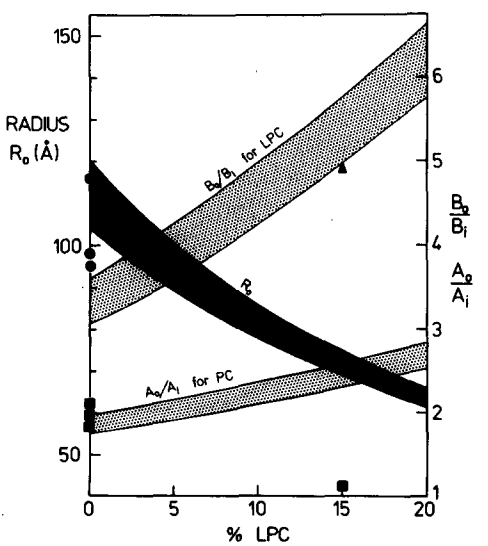

Fig. 1. Vesicle radius R_0, and outer/inner lipid distributions (A_0/A_i and B_0/B_i) in mixed phosphatidylcholine/lysophosphatidylcholine vesicles as a function of composition. The radius is given by Eqn. 7 while the distributions are calculated from Eqn. 6. The shaded regions indicate the range of theoretical predictions. Note that for no asymmetry the curves for A_0/A_i and B_0/B_i would coincide. Experimental values: •, vesicle radii R_0 [11,12,20]; ■, A_0/A_i [12,16,32]; ▲, B_0/B_i [32]; see also Results.

taining 10% lysophosphatidylcholine (i.e. $F = 0.9$). The found $[(A_0 + B_0)/(A_i + B_i)] = f/(1 - f) = 2.4 \pm 0.05$. Our calculated value is in the range 2.25—2.52 for l_c in the range 17.0—17.5 Å, in agreement with their value. However, they then calculate R_0 by taking a total bilayer thickness of 35 Å and so obtain $R_0 =$ 107 Å. As shown above this procedure is not correct since the headgroup areas, taken at the ends of the polar groups, are different in the outer and inner monolayers. Using a hydrocarbon thickness of approx. 29 Å, their measured value of $[(A_0 + B_0)/(A_i + B_i)]$ yields $R_0 \approx 82$ Å, which then agrees with our values of 78—86 Å.

Mandersloot et al. [30] measured a thickness decrease in phosphatidylcholine/lysophosphatidylcholine liposomes as the fraction of lysophosphatidylcholine increased. They found a decrease in total bilayer thickness from 39 Å (pure phosphatidylcholine) to 35 Å (25% lysophosphatidylcholine). For a planar bilayer the hydrocarbon thickness, given by $2\bar{v}/\bar{a}$, should equal $2 \times 1063/71.7 \approx 30$ Å for pure phosphatidylcholine, and $2(1063 \times 0.75 + 531 \times 0.25)/71.7 \approx 26$ Å for 25% lysophosphatidylcholine. This decrease of 4 Å is in agreement with that measured. We find a similar decrease (results not shown) of the hydrocarbon thicknes in phosphatidylcholine/lysophosphatidylcholine vesicles. Mandersloot et al. [30] also report that vesicles with more than 50% lysophosphatidylcholine do not form. We find similar behaviour in that for vesicles with more than a critical fraction of lysophosphatidylcholine packing constraints cannot be fulfilled and a spherical vesicle cannot form (i.e. the theory predicts that they will shrink to micelles probably of cylindrical shape [31]). This critical fraction is very sensitive to l_c but should be close to 30—40% lysophosphatidylcholine.

In a later paper, de Kruijff et al. [32] measured the transbilayer distributions of phosphatidylcholine and lysophosphatidylcholine in cosonicated vesicles containing 15% lysophosphatidylcholine ($F = 0.85$). They found the outside/inside ratio of lysophosphatidylcholine (B_0/B_i) to be about 4.9. This agrees with our values of 5.0—5.8 though it should be noted that the theoretical value for B_0/B_i is very sensitive to the values chosen for l_c and D. The experimental value of B_0/B_i is likewise very sensitive to the error in measuring B_i, since B_i is so small (about 3% of the total lipid). A less sensitive measure is the fraction of total lysophosphatidylcholine in the outer monolayer $[B_0/(B_0 + B_i)]$. De Kruijff et al. [32] find 83% lysophosphatidylcholine outside, compared with our values of 83—85%. Another experiment [33] found 80—90% lysophosphatidylcholine outside. In the same paper, de Kruijff et al. give results which indicate the outside/inside ratio of phosphatidylcholine (A_0/A_i) in these same vesicles ($F = 0.85$) to be approx. $0.44/0.41 = 1.07$. We calculate $A_0/A_i =$ 2.3—2.6, in disagreement with this experimental result. Taken together, de Kruijff's two results give $[(A_0 + B_0)/(A_i + B_i)] = f/(1 - f) = 1.3$ which is irreconcilable with their earlier [16] value of 2.4, which admittedly used 10% lysophosphatidylcholine rather than 15% lysophosphatidylcholine. Further, taking the most charitable choice for the hydrocarbon thickness of $t = 29$ Å together with their value of $f/(1 - f) = 1.3$ yields $R_0 \approx 200$ Å in contradiction with the observation that vesicle radii decrease as lysophosphatidylcholine is added [16].

We now discuss the general features of our results and see how they can be

explained using simple ideas. From Fig. 1 we see that the radius of the vesicles decreases as the amount of lysophosphatidylcholine increases. This is simply a result of the different packing properties of lysophosphatidylcholine and phosphatidylcholine. The former can pack into highly curved micellar structures, while phosphatidylcholine can only pack into a much less curved vesicular structure. A mixture of these lipids will therefore pack into a structure of intermediate curvature, in this case, a vesicle of smaller radius than that for phosphatidylcholine.

There are two effects that determine the outside/inside distribution of the lipids: one due to vesicle size and the other due to asymmetry. We discuss these in turn. Since the radius decreases with increasing lysophosphatidylcholine and the head group areas are roughly the same for the two lipids, the outside/inside ratio even for a symmetric distribution will increase, since the ratio of outer to inner vesicle surface areas increases as R_0 falls. In addition, any asymmetry in which one species preferentially packs into, say, the outer monolayer will further effect the distributions.

For the phosphatidylcholine/lysophosphatidylcholine system the more tapered shape of lysophosphatidylcholine causes it to go preferentially to the outside, which gives more lysophosphatidylcholine and less phosphatidylcholine in the outer monolayer than would otherwise be the case. For phosphatidylcholine these two effects (radius decrease leading to an increase in A_0/A_i, and asymmetry leading to a decrease in A_0/A_i) almost cancel out; the net effect is that the outside/inside ratio of phosphatidylcholine (A_0/A_i) increases only slowly as more lysophosphatidylcholine is added (Fig. 1). But for lysophosphatidylcholine both effects work in the same direction so that the outside/inside ratio of lysophosphatidylcholine (B_0/B_i) increases very rapidly (Fig. 1). It is worth noting that if there were no asymmetry but only a vesicle size change the values of A_0/A_i and B_0/B_i would be equal at all mol fractions F and vesicle radii R_0.

Phosphatidylcholine/cholesterol

Our calculations for phosphatidylcholine/cholesterol vesicles are shown in Fig. 2 which exhibits the variation of vesicle radius R_0, and transbilayer distribution of both phosphatidylcholine (A_0/A_i) and cholesterol (B_0/B_i) as the mol fraction of cholesterol is increased to 50%. Again the geometric properties of the molecules were taken from Table I.

As can be seen, the values for the mixed vesicle radii agree well with the varied experimental values shown. Johnson [11] used hydrodynamic methods to obtain the vesicle radius (note that his vesicles contained a small amount, approx. 4%, of phosphatidic acid which may have changed the vesicle size slightly). Gent and Prestegaard [20] used gel partition chromatography and obtained slightly larger radii. Newman and Huang [34] obtained values for R_0 of 102 Å, 105 Å and 111 Å for 0%, 9.5% and 22.5% cholesterol, respectively, using hydrodynamic methods, slightly less than Johnson's values.

The behaviour of the transbilayer distribution of phosphatidylcholine depends on the type of phosphatidylcholine used. For phosphatidylcholine with *cis*-double bonds (egg phosphatidylcholine, soy phosphatidylcholine, $18:1_c/18:1_c$ phosphatidylcholine) there is an abrupt increase in A_0/A_i above

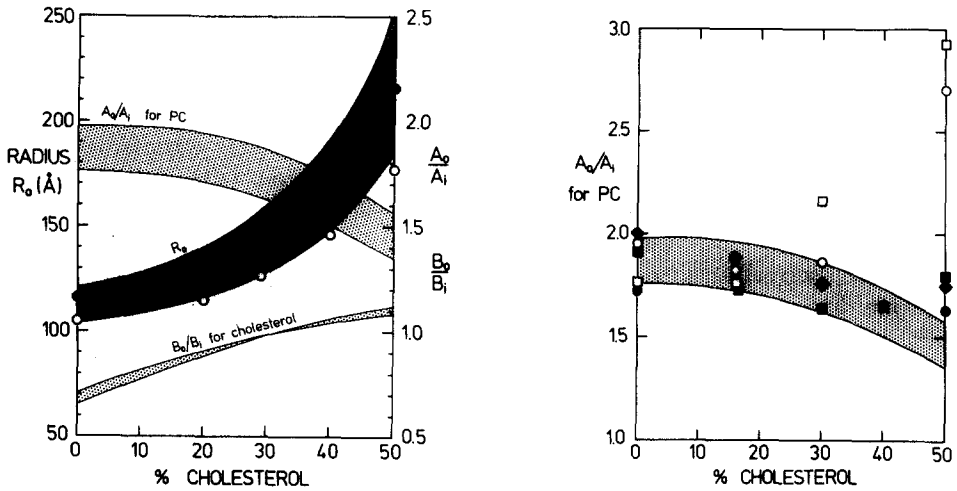

Fig. 2. Vesicle radii R_0 and distributions A_0/A_i and B_0/B_i for mixed phosphatidylcholine/cholesterol vesicles; details as in Fig. 1. Experimental values [20,11] shown for radii R_0 only (●, ○). See also Fig. 3.

Fig. 3. Outer/inner distribution of phosphatidylcholine in mixed phosphatidylcholine/cholesterol vesicles. Theoretical values are represented by the shaded region as in Fig. 2. Experimental values [35] are: ●, for 18 : 0/18 : 0 phosphatidylcholine; ■, 16 : 0/16 : 0 phosphatidylcholine; ◆, 18 : 1_t/18 : 1_t phosphatidylcholine; □, 18 : 1_c/18 : 1_c phosphatidylcholine, and ○, egg phosphatidylcholine. Note that the open data points are for lipids containing *cis*-double bonds.

about 30% cholesterol [35,36]. For saturated phosphatidylcholines or phosphatidylcholines with *trans*-double bonds, however, there is only a small change or even a decrease in A_0/A_i as the fraction of cholesterol is increased. Our results shown in Fig. 3, agree quite well with all cases for up to 25% cholesterol, and with the saturated and *trans*-double bond phosphatidylcholine up to 50% cholesterol.

There have been no direct measurements of the transbilayer distribution of cholesterol in these mixed vesicles. De Kruijff et al. [35] calculate the distribution indirectly, from their measured values of the transbilayer distribution of phosphatidylcholine, assigning values for the headgroup area of phosphatidylcholine, cholesterol and a phosphatidylcholine-cholesterol complex. For $F = 0.5$, they obtain values for B_0/B_i in the range 0.82—1.38 for various phosphatidylcholines, compared with our values in the range 1.08—1.13. These measurements give 45—58% of the total cholesterol outside, compared with our values of 52—53%.

The general features of Fig. 2 are explicable in much the same terms as Fig. 1. Because cholesterol is a frayed structure [4] the vesicles increase in size as more cholesterol is added. This is just the opposite case to that in Fig. 1, in which lysophosphatidylcholine is more tapered than phosphatidylcholine.

Because the vesicles increase in size, both A_0/A_i and B_0/B_i should decrease as the fraction of cholesterol is increased in the absence of asymmetry. There is, however, some asymmetry, with phosphatidylcholine now preferentially packing in the outer monolayer. For phosphatidylcholine this means that A_0/A_i does not decrease as rapidly as it would for a symmetric distribution. For small amounts of cholesterol it is more favourable for cholesterol to pack mostly in

the inner monolayer (i.e. $B_0/B_i < 1$). As the fraction of cholesterol increases, the vesicles increase in size so the tendency for cholesterol to pack in the inner monolayer decreases (cf. Eqn. 2); the net effect is that B_0/B_i increases slowly as more cholesterol is added.

Our results also show a small monotonic increase in hydrocarbon region thickness from approx. 30 Å (pure phosphatidylcholine) to approx. 32 Å (50% cholesterol) consistent with experiment [37]. This thickness increase is a result of the frayed nature of the cholesterol molecule which causes the phosphatidylcholine to become more tapered, i.e. the phosphatidylcholine chains are forced to straighten out and consequently to become less fluid like [4].

Measurements of the size distribution of mixed phosphatidylcholine/cholesterol vesicles indicate that the standard deviation in vesicle size is about 20% [62,63]. This is significantly higher than our theoretical estimate of a few percent (cf. Eqn. 10) which we stress gives only a lower bound for σ_R. However, we note that different methods of vesicle preparation yield different size distributions. In particular, vesicles prepared without sonication have a much narrower spread in size, of the order of a few percent [64].

The sudden upturn in A_0/A_i for *cis*-unsaturated phosphatidylcholine above 25% cholesterol (Fig. 3) could be accounted for by a small increase in the effective head group length D_A of phosphatidylcholine, and we note that whereas Brown and Seelig [39] conclude that for saturated dipalmitoylphosphatidylcholine there are only slight changes in headgroup conformation as the fraction of cholesterol is increased, Verma and Wallach [38] and Oldfield et al. [58] measured significant changes in headgroup configuration and motion of *cis*-unsaturated phosphatidylcholines above 20% cholesterol. Such specific interactions have also been observed in monolayer studies [40,41].

Packing, flip-flop, and bilayer shape changes

The theory and results presented above apply to equilibrium vesicles. We now explore the possibility that vesicles can deform so as to relieve the unfavourable packing strains introduced by the non-equilibrium incorporation of foreign molecules (thereby reducing one of the driving forces of flip-flop [32,42]). Qualitatively we expect packing strains to arise when the guest lipids have a larger critical (vesicle) radius than the host lipids, for then packing constraints cannot be satisfied even if the vesicles deform. Such incorporation should therefore induce flip-flop. These ideas are in agreement with observations that the incorporation of low radius of curvature lipids into vesicles preformed with higher radius of curvature lipids (e.g. 16 : 0 lysophosphatidylcholine into $18 : 1_c/18 : 1_c$ phosphatidylcholine vesicles [32] or dimyristoylphosphatidylcholine into dioleoylphosphatidylcholine vesicles [42]) did not increase the flip-flop rate; but when the guest lipid had the larger radius of curvature there was more than a two-fold increase in the rate of transbilayer lipid movement.

As an example of how unfavourable packing strains can be relieved on incorporation of foreign lipids into vesicles, we have considered the experimental results of de Kruijff et al. [32] who found that egg phosphatidylcholine vesicles when incubated with lysophosphatidylcholine were able to incorporate at least 13% of lysophosphatidylcholine in the outer vesicle monolayer without

inducing flip-flop. We have verified that if the vesicles become elongated during incorporation of lysophosphatidylcholine, the resulting vesicles may still have the same number of phosphatidylcholine molecules on the inner and outer monolayers as well as an additional 10% or more lysophosphatidylcholine in the outer monolayer. Thus, for example, a tubular vesicle with hemispherical ends (of diameter approx. 130 Å and length approx. 400 Å) could accommodate approx. 13% lysophosphatidylcholine in the outer monolayer, mainly in the hemispherical end regions, with no packing strains being induced on either phosphatidylcholine or lysophosphatidylcholine in either monolayers, and therefore with little tendency for induced flip-flop to occur for either species. A more likely shape would be intermediate between a tubular and a prolate ellipsoidal structure, still with an axial ratio of approx. 3. More elongated vesicles could accommodate up to 18% lysophosphatidylcholine in the outer monolayer before packing strains become unfavourable.

Discussion

Our quantitative study of mixed phosphatidylcholine/lysophosphatidylcholine and phosphatidylcholine/cholesterol vesicles has successfully described the variations in vesicle radii and asymmetric lipid distributions as a function of composition. That a theory formulated primarily in terms of lipid shapes applies to these very different lipids indicates that specific lipid interactions are, for the most part, not important. The unusual lipid distributions observed with *cis*-unsaturated phosphatidylcholine when more than 30% cholesterol is present is the only instance where the theory could not account for the expermental data. However, as argued in the text and supported by some experimental evidence, it is likely that here specific interactions involving the bulky *cis*-double bond of phosphatidylcholine could cause the head group conformation of phosphatidylcholine to change in such a way as to bring the theoretical predictions in line with the measured results. The theory, therefore, has its weakness in not being generally applicable to all types of lipid-lipid interactions; but its strength lies in being able to pinpoint where specific interactions have to be invoked as well as giving some indication of how these affect lipid packing.

The present theory describes the equilibrium properties of mixed lipid vesicles. For the most highly tapered lipid, lysophosphatidylcholine, it is easily possible to find very large transbilayer asymmetries, arising solely from packing effects. Such consideration will not always apply to biological membranes which are essentially planar. Only when the local radius of curvature is small, as may happen during pinocytosis and blebbing, or in microvilli and alveoli, will these effects become important, leading to large local asymmetrics and possible separation of lipid and protein species between the curved and planar regions of the membranes [59]. Another important difference between lipid vesicles and biological membranes is the existence of a resting potential across membranes which is also likely to be correlated with the asymmetry of the charged species [44]. Thus the asymmetries observed in biological membrane [45—47] cannot be explained in terms of the theory. Nevertheless, the theory can at least rationalise in terms of packing compatibilities why certain lipid species are

often found together, while others are rarely found together, on the same side of a biological membrane of lipid bilayer. For example, the concepts of packing explain the preference of cholesterol for phosphatidylcholine rather than phosphatidylethanolamine in a mixture of these three lipids [48], since phosphatidylcholine, being more tapered than phosphatidylethanolamine, can more easily accommodate the slightly frayed shape of cholesterol than can phosphatidylethanolamine. Further, the packing incompatability of phosphatidylethanolamine and cholesterol implies that when mixed these should not form vesicles, as observed [49]. Another study of preferential lipid distributions [50] has shown that cholesterol prefers sphingomyelin most, with phosphatidylcholine next, and showing least preference for phosphatidylethanolamine. This indicates that sphingomyelin is more tapered than phosphatidylcholine, and so should form smaller vesicles, and pack preferentially into the outside of sphingomyelin/phosphatidylcholine vesicles, as observed [43]. Finally, the ability of cholesterol to 'neutralise' the lytic activities of lysophosphatidylcholine can be readily understood in terms of the mutual accommodation of these two differently shaped lipids (the one tapered and the other frayed), as can their ability to form bilayers when mixed [65].

On a final note: the assumption, adopted herein, that each lipid species in a mixed system has the same effective chain length l_c is a severe one. In fact, the sensitivity of the theoretical results to chain length indicates its fundamental role. We propose to incorporate hydrocarbon chain energetics into the present formalism and so facilitate the consideration of chain length differences in a natural way. A rigorous discussion of lateral phase separations in mixed lipid systems may then be possible. Finally, we note that the packing of proteins in a lipid environment may also be discussed in terms of these packing ideas [7,61], although such applications remain, as yet, unexplored quantitatively.

Appendix

We present here more detailed discussion of the theory outlined in section 2. To a sufficient approximation the free energy of a two component spherical vesicle of aggregation number N is given by a slightly rearranged version of Eqn. 31 of Ref. 6:

$$G = N\mu_N^0 = 2N\gamma(Fa_A + (1-F)a_B) + N\gamma\left(\frac{(1-f)}{R_i}[X_iD_Aa_A + (1-X_i)D_Ba_B]\right.$$
$$\left. - \frac{f}{R_0}[X_0D_Aa_A + (1-X_0)D_Ba_B]\right) + NkT(f[X_0 \ln X_0 + (1-X_0)\ln(1-X_0)]$$
$$+ (1-f)[X_i \ln X_i + (1-X_i)\ln(1-X_i)]) \qquad \text{(A1)}$$

where the notation was defined in Eqn. 1.

In Eqn. A1 the three contributions are, respectively, the free energy of the two hydrocarbon-water interfaces, a correction due to finite curvature and head group length and, last, the entropy of mixing of lipids in each monolayer, here assumed to be ideal.

A rigorous description of vesicle sizes must follow from solution thermodynamics [5,6]. The equilibrium between monomers and aggregates gives the

mol fraction of lipids in vesicles of aggregate number N:

$$X_N = NX_1^N \exp[-N(\mu_N^0 - \mu_1^0)/kT]$$

Thus, choosing N (or R_0), one minimises G (Eqn. A1) subject to packing constraints to find X_N as a function of N. Generally the distributions of X_N is sharply peaked, N (average) $\approx N$ (most probable) and the corresponding vesicle radius is approx. R_0^c. The procedure of simply minimising G with respect to all variables, including N, yields essentially identical results, especially since the monomer concentration is negligible, and we adopt it here.

Free energy minimisation

We shall return presently to discuss the approximations inherent in Eqn. A1. First, however, we discuss the minimisation of the free energy subject to packing constraints. These constraints, which simply express the conservation of hydrocarbon volume and area, are

$$4\pi(R_0^c)^2 = Nf[X_0 a_A + (1 - X_0)a_B] = Nf\bar{a} \tag{A2}$$

$$4\pi R_i^2 = N(1 - f)[X_i a_A + (1 - X_i)a_B] \tag{A3}$$

$$\frac{4\pi}{3}[(R_0^c)^3 - R_i^3)] = N[Fv_A + (1 - F)v_B] \tag{A4}$$

$$\frac{4\pi}{3}[(R_0^c)^3 - (R_0^c - l_c)^3] = Nf[X_0 v_A + (1 - X_0)v_B] = Nf\bar{v} \tag{A5}$$

The vesicles which preferentially form are adequately described as being those which minimise * G subject to the constraints of Eqns. A2—A5; the expression for X_i given in Eqn. 1, should also be regarded as a 'constraint'. The full minimisation problem is difficult since once v_A, v_B, a_A, a_B, l_c, D_A, D_B, and F are specified G is still a function of six variables (N, R_0, R_i, f, X_0, X_i). An approximate procedure follows, however, from the observations that G is most strongly dependent upon N and X_0 and that asymmetries ($\Delta X_0 = X_0 - F$) are usually small. (Note that whilst ΔX_0 may be small, A_0/A_i may be very large). Then we may expand G about $G_F = G(X_0 = F)$:

$$G \approx G_F + \left(\frac{\partial G}{\partial X_0} + \frac{\partial G}{\partial N}\frac{\partial N}{\partial X_0} + \ldots\right)\Delta X_0 + \frac{1}{2}\frac{\partial^2 G}{\partial X_0^2}(\Delta X_0)^2 + \ldots \tag{A6}$$

Terms omitted here, such as

$$\frac{\partial G}{\partial R_0}\frac{\partial R_0}{\partial X_0}, \frac{\partial G}{\partial f}\frac{\partial f}{\partial X_0},$$

etc., are of secondary importance. In addition the entropy of mixing terms in G can be linearised: $\ln(X_0) = \ln(F + \Delta X_0) \approx \ln F + \Delta X_0/F + \ldots$, etc. This approximation is justified a posteriori by the solution to the full non-linear problem, discussed below. The terms in Eqn. A6 can be calculated approximately using Eqns. 1, A1 and A5 directly or, more rigorously, using Jacobians. The mini-

* This could well be a local minimum; we have not considered lamellar phases which may be thermodynamically preferred.

misation condition is then simply $dG/dX_0 = 0$ and yields

$$X_0 \approx F - \left[\frac{\partial G}{\partial X_0} + \frac{\partial G}{\partial N}\frac{\partial N}{\partial X_0}\right] \Big/ \frac{\partial^2 G}{\partial X_0^2} \tag{A7}$$

from which Eqn. 2 follows.

The equilibrium vesicle size and lipid distribution are then found by solving Eqns. 2 and A2—A5. These are a set of simultaneous non-linear equations for N, R_0, R_i, X_0 and f (X_i being given by Eqn. 1) and can be solved by iterating from $X_0 \approx F$; a few iterations yield convergence to one part in 10^5. Results given by Eqn. 6 are essentially identical. We remark parenthetically that Eqn. 7 follows from Eqns. A2 and A5.

The rigorous minimisation procedure does not require the linearisation of the entropy of mixing and employs the method of Lagrange Multipliers. The resultant system of six non-linear and five linear equations must be solved numerically. We have done this for the phosphatidylcholine/lysophosphatidylcholine and phosphatidylcholine/cholesterol systems and find only a few percent difference from the approximate procedure described above and used in the results section.

Approximations

The expression for the free energy given by Eqn. A1 includes two major approximations: (1) optimal head group packing and, (2) ideal mixing. Contributions to G due to non-optimal head group packing in the outer monolayer produces extra terms in Eqn. A1 of the form:

$$Nf\gamma(X_0 a_{A0}(1 - a_A/a_{A0})^2 + (1 - X_0)\, a_{B0}(1 - a_B/a_{B0})^2)$$

where a_{A0} and a_{B0} are the actual head group areas of lipids in the outer monolayer as distinct from the optimal areas a_A, a_B. Such terms have been neglected here since they are of lower order than the entropy of mixing. Their inclusion considerably complicates the analysis yet produces little effect on the final results and so we do not pursue this point here. The approximation $R_0 \approx R_0^c$ in Eqns. A2, A4, and A5 is tantamount to that of optimal packing.

The assumption of ideal mixing, even for lipids as different as phosphatidylcholine, lyosphosphatidylcholine and cholesterol is, surprisingly, reasonable. This follows from the neglect of specific lipid-lipid interactions which, at first sight, might be regarded as a weak point of the theory but is, in fact, its strength: if a reasonable description (i.e. one that agrees with experiment) of vesicle sizes and lipid distributions follows without invoking specific lipid interactions, then this provides strong evidence that such effects need not be implicated, contrary to common assumption. The lipid tails are here assumed to interact only via an excluded volume ('hard sphere' repulsion) effect. That mean field theory descriptions [19] of the liquid-solid phase transition in lipid bilayers are so succesful further argues for our neglect of specific lipid-lipid interations. The experimental observation [58] that there is no evidence for long-lived phosphatidylcholine/cholesterol complexes in mixed liposomes also suggests that specific interactions may not be important. Given only excluded volume interactions the assumption of ideal mixing is reasonable, even for lipids with very different hydrocarbon and head group areas, such as those con-

sidered herein. Computer simulation of equimolar mixtures of hard spheres with diameters in the ratio 3 : 1 indicate near ideality [55]; non-ideal corrections to the entropy of mixing are less than 5% at low pressure and never exceed 30%, even near solidification. Calculations in two dimensions are unavailable, but, since hard rods (i.e. hard spheres in one dimension) are exactly ideal [56], we can safely presume hard particles to be more ideal in two dimensions than in three. This could be easily verified using, for instance, scaled particle theory [57] in two dimensions.

References

1 Evans, E.A. (1974) Biophys. J. 14, 923—931
2 Helfrich, W. (1973) Z. Naturforsch. 28c, 693—703
3 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4457—4461
4 Israelachvili, J.N. and Mitchell, D.J. (1975) Biochim. Biophys. Acta 389, 13—19
5 Israelachvili, J.N., Mitchell, D.J. and Ninham, B.W. (1976) J. Chem. Soc. Faraday Trans. II 72, 1525—1568
6 Israelachvili, J.N., Mitchell, D.J. and Ninham, B.W. (1977) Biochim. Biophys. Acta 470, 185—201
7 Israelachvili, J.N. (1977) Biochim. Biophys. Acta 469, 221—225
8 Deuling, H.J. and Helfrich, W. (1976) Biophys. J. 16, 861—868
9 Small, D.M. (1967) J. Lipid Res. 8, 551—557
10 Reiss-Husson, F. (1967) J. Mol. Biol. 25, 363—382
11 Johnson, S.M. (1973) Biochim. Biophys. Acta 307, 27—41
12 Huang, C. and Mason, J.T. (1978) Proc. Natl. Acad. Sci. U.S. 75, 308—310
13 Levine, Y.K. and Wilkins, M.F.H. (1971) Nat. New Biol. 230, 69—72
14 Wilkins, M.H., Blaurock, A.E. and Engelman, D.M. (1971) Nat. New Biol. 230, 72—76
15 Chrzeszczyk, A., Wishnia, A. and Springer, C.S., Jr. (1977) Biochim. Biophys. Acta 470, 161—169
16 De Kruijff, B., Cullis, P.R. and Radda, G.K. (1975) Biochim. Biophys. Acta 406, 6—20
17 Perrin, J.H. and Saunders, L. (1964) Biochim. Biophys. Acta 84, 216—217
18 Tanford, C. (1973) The Hydrophobic Effect, John Wiley and Sons, New York
19 Marcelja, S. (1974) Biochim. Biophys. Acta 367, 165—176
20 Gent, M.P.N. and Prestegaard, J.H. (1974) Biochemistry 13, 4027—4033
21 Crowfoot, D. (1944) Vitam. Horm. 2, 409—461
22 Phillips, M.C., Ladbrooke, B.D. and Chapman, D. (1970) Biochim. Biophys. Acta 196, 35—44
23 Yeagle, P.L., Hutton, W.C., Huang, C. and Martin, R.B. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3477—3481
24 Seelig, J., Gally, H.-U. and Wohlgemuth, R. (1977) Biochim. Biophys. Acta 467, 109—119
25 Franks, N.P. (1976) J. Mol. Biol. 100, 345—358
26 Worcester, D.L. and Franks, N.P. (1976) J. Mol. Biol. 100, 359—378
27 Hauser, H., Phillips, M.C., Levine, B.A. and Williams, R.J.P. (1976) Nature 261, 390—394
28 Huang, C. (1977) Lipids 12, 348—356
29 Chatterjee, N. and Brockerhoff, H. (1978) Biochim. Biophys. Acta 511, 116—119
30 Mandersloot, J.G., Reman, F.C., van Deenen, L.L.M. and de Gier, J. (1975) Biochim. Biophys. Acta 382, 22—26
31 Robinson, N. (1961) J. Pharm. Pharmacol. 13, 53—57
32 De Kruijff, B., van der Besselaar, A.M.M.P. and van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 465, 443—453
33 Van den Besselaar, A.M.H.P., van den Bosch, H. and van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 465, 454—465
34 Newman, G.C. and Huang, C. (1975) Biochemistry 14, 3363—3370
35 De Kruijff, B., Cullis, P.R. and Radda, G.K. (1976) Biochim. Biophys. Acta 436, 729—740
36 Huang, C., Sipe, J.P., Chow, S.T. and Martin, R.B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 359—362
37 Lecuyer, H. and Dervichian, D.G. (1969) J. Mol. Biol. 45, 39—57
38 Verma, S.P. and Wallach, D.F. (1973) Biochim. Biophys. Acta 330, 122—131
39 Brown, M.F. and Seelig, J. (1978) Biochemistry 17, 381—384
40 Demel, R.A., van Deenen, L.L.M. and Pethica, B.A. (1967) Biochim. Biophys. Acta 135, 11—19
41 Demel, R.A. and Joos, P. (1968) Chem. Phys. Lipids 2, 35—46
42 De Kruijff, B. and Wirtz, K.W.A. (1977) Biochim. Biophys. Acta 468, 318—326
43 Berden, J.A., Barker, R.W. and Radda, G.K. (1976) Biochim. Biophys. Acta 375, 186—208
44 McLaughlin, S. and Harary, H. (1974) Biophys. J. 14, 200—208

45 Bretscher, M.S. (1973) Science 181, 622—629
46 Nilsson, O. and Dallner, G. (1975) FEBS Lett. 58, 190—193
47 Tsai, K.W. and Lenard, J. (1975) Nature 253, 554—555
48 Van Dijck, P.W.M., de Kruijff, B., van Deenen, L.L.M., de Gier, J. and Demel, R.A. (1976) Biochim. Biophys. Acta 455, 576—588
49 Patzer, E.J., Wagner, R.R. and Barenholz, Y. (1978) Nature 274, 394—395
50 Demel, R.A., Jansen, J.W.C.M., van Dijck, P.W.M. and van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 465, 1—10
51 Brown, M.F. and Seelig, J. (1977) Nature 269, 721—723
52 Papahadjopoulos, D., Poste, G., Schaeffer, B.E. and Vail, W.J. (1974) Biochim. Biophys. Acta 352, 10—28
53 Papahadjopoulos, D., Vail, W.J., Jacobsen, K. and Poste, G. (1975) Biochim. Biophys. Acta 394, 483—491
54 Hall, J.E. and Simon, S.A. (1976) Biochim. Biophys. Acta 436, 613—616
55 Alder, B.J. (1964) J. Chem. Phys. 40, 2724—2730
56 Chan, D.Y.C., Mitchell, D.J., Ninham, B.W. and Pailthorpe, B.A. (1978) J. Chem. Soc. Faraday Trans. II, 74, 2050—2061
57 Stillinger, F.H. (1973) J. Solution Chem. 2, 141—151
58 Oldfield, E., Meadows, M., Rice, D. and Jacobs, R. (1978) Biochemistry 17, 2727—2740
59 Shukla, S.D., Berriman, J., Coleman, R., Finean, J.T. and Mitchell, R.H. (1978) FEBS Lett. 90, 289—292
60 De Bernard, L. (1958) Bull. Soc. Chim. Biol. 40, 161—170
61 Israelachvili, J.N. (1978) in Light-Transducing Membranes (Deamer, D., ed.), pp. 91—107, Academic Press, New York
62 Watts, A., Marsh, D. and Knowles, P.F. (1978) Biochemistry 17, 1792—1801
63 Forge, A., Knowles, P.F. and Marsh, D. (1978) J. Membrane Biol. 41, 249—263
64 Brunner, J., Skrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta 455, 322—331
65 Purdon, A.D., Hsia, J.C., Pinteric, L., Tinker, D.O. and Rand, R.P. (1975) Can. J. Biochem. 53, 196—206