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COOPERATIVITY OF THE PHASE TRANSITION IN SINGLE- AND MULTIBILAYER LIPID VESICLES

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Summary

The effect of membrane morphology on the cooperativity of the ordered-fluid, lipid phase transition has been investigated by comparing the transition widths in extended, multibilayer dispersions of dimyristoyl phosphatidylcholine, and also of dipalmitoyl phosphatidylcholine, with those in the small, single-bilayer vesicles obtained by sonication. The electron spin resonance spectra of three different spin-labelled probes, 2,2,6,6-tetramethylpiperdine-*N*-oxyl, phosphatidylcholine and stearic acid, and also 90° light scattering and optical turbidity measurements were used as indicators of the phase transition. In all cases the transition was broader in the single-bilayer vesicles than in the multibilayer dispersions, corresponding to a decreased cooperativity on going to the small vesicles. Comparison of the light scattering properties of centrifuged and uncentrifuged, sonicated vesicles suggests that these are particularly sensitive to the presence of intermediate-size particles, and thus the spin label measurements are likely to give a more reliable measure of the degree of cooperativity of the small, single-bilayer vesicles. Application of the Zimm and Bragg theory ((1959) J. Chem. Phys. 31, 526—535) of cooperative transitions to the two-dimensional bilayer system shows that the size of the cooperative unit, $1/\sqrt{\sigma}$, is a measure of the mean number of molecules, per perimeter molecule, in a given region of ordered or fluid lipid at the centre of the transition. From this result it is found that it is the vesicle size which limits the cooperativity of the transition in the small, single-bilayer vesicles. The implications for the effect of membrane structure and morphology on the cooperativity of phase transitions in biological membranes, and for the possibility of achieving lateral communication in the plane of the membrane, are discussed.

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Abbreviation: TEMPO, 2,2,6,6-tetramethyl-piperidine-*N*-oxyl.

Introduction

The possible importance of the lipid phase transition [1,2], and of lateral lipid phase separation [3], in the functioning of biological membranes is now well established (for a review see e.g. ref. 4). Recently the possible functional significance of the interfacial lipid, which exists during the phase transition or phase separation, has also been emphasized [5—7]. Lipid phase transitions or phase separations have thus been implicated in carrier-mediated transport [3, 8], enzymatic activity [9—11] ion permeability [6,7] and penetration of proteins into the membrane [5].

A topic of considerable interest, concerning both lipid bilayers and biological membranes, is the degree of cooperativity of the phase transition [6,12,13] which is also intimately connected with the size of the cooperative unit which undergoes the transition [6,14]. The degree of cooperativity affects the sharpness of the transition, i.e. the range of lateral phase separation over which ordered and fluid lipid co-exist. Thus the effects of a possible triggering of the phase transition [2] will depend on the degree of cooperativity of the transition, and it has also been shown that during the transition or lateral phase separation, the concentration of interfacial lipid depends sensitively on the degree of cooperativity [6]. Of perhaps even greater importance is the possibility of lateral communication within the plane of the membrane being effected by the phase transition; the effectiveness of such a mechanism would be sensitively dependent on the size of the cooperative unit undergoing the transition.

In biological membranes the way in which the size of the cooperative unit is limited by the morphology of the membrane surface, or by other membrane components and different regions of lipid, will thus be of considerable importance. This will be especially so in the case where lipid regions have been shown to be differentially segragated about membrane protein complexes [15]. In the present paper we report the results of a study of the effect of membrane morphology on the cooperativity of the phase transition in phospholipid bilayers.

Materials and Methods

Dimyristoyl phosphatidylcholine was obtained from Calbiochem and from Sigma and dipalmitoyl phosphatidylcholine was obtained from Calbiochem and from Fluka. All lipids were shown to be pure by thin-layer chromatography in both acidic and basic solvent systems [16]. The spin label TEMPO I was prepared according to the method of Rozantzev and Neiman [17]. Spin-labelled phosphatidylcholine II (12,3) was prepared by condensing the stearic acid spin label (12,3) onto 1-palmitoyl lysophosphatidylcholine, according to the method of Hubbell and McConnell [18]. Stearic acid spin label III (12,3) was obtained from Syva. Lipid dispersions were all prepared in a standard buffer which was 0.1 M KCl/0.01 M Tris · HCl adjusted to pH 8.0.

Electron spin resonance measurements. For measurements with the partitioning of the TEMPO spin label I, 25 mg of phospholipid was dispersed in 1 ml of 10^{-4} M solution of TEMPO in buffer, by shaking above the phase

transition temperature using a vortex mixer. Dispersions were either used directly as prepared in this way, or after subsequent sonication. Sonication was performed in 25-s bursts using a 1/8 inch titanium microtip, until further sonication produced no more clarification of the suspension, normally after 5 min total sonication time. During sonication the sample was thermostatted in a water-jacketted sample tube at a temperature 10°C above the phase transition. After sonication the sample was centrifuged at 105 000 × *g* for 10 min to remove titanium fragments and undispersed phospholipid. Under these conditions the lipid dispersion is converted almost entirely into homogeneous, single-bilayer vesicles [6]. Analysis of the extracted lipid after sonication, by thin-layer chromatography as described above, yielded no evidence for any chemical degradation of the phospholipid during sonication or centrifugation. Phosphate analysis [19] of the lipid dispersion after centrifugation revealed that less than 10% of the lipid was lost from the clear supernatant during this process.

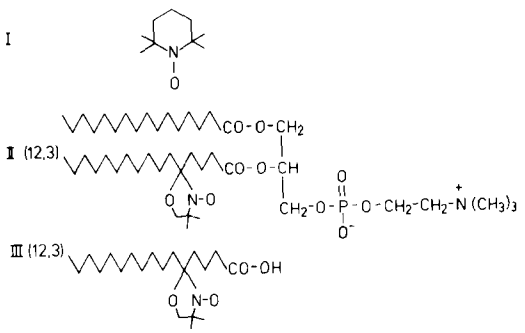
For experiments using the lipid spin labels II (12,3) or III (12,3) 1% by weight of the spin label was dissolved together with the lipid in chloroform. The chloroform was evaporated off under nitrogen and was then placed under vacuum overnight. The anhydrous lipid was then dispersed in buffer as described above.

ESR measurements were made with a Varian E-12 9 GHz spectrometer equipped with a nitrogen gas flow, temperature regulation system. Samples were contained in sealed-off 100-μl capillary pipettes accomodated within standard 4-mm quartz ESR tubes containing silicon oil for thermal stability. Heating rates of approx. 15°C per h were used; measurements were made at fixed temperatures after the temperature had stabilized. Temperatures were measured with a thermocouple placed just above the cavity within the quartz ESR tube. No change in the physical appearance of the clear sonicated samples was observed during the experiment, whereas some settling-out of the lipid in the unsonicated dispersions was unavoidable. No appreciable hysteresis was observed in the measured parameters on cooling the sample.

Optical measurements. Lipid dispersions were prepared essentially as described above for the ESR measurements, except that the lipid concentration was 1 mg/ml for the light scattering measurements and 2 mg/ml for the absorbance measurements. Sonicated dispersions were also prepared as described above at a lipid concentration of 1 mg/ml for light scattering and 4 mg/ml for absorbance measurements. 90° light scattering was recorded continuously at 550 nm using a Fluorispec spectrofluorimeter with a nitrogen gas flow thermostat system programmed to change the temperature at a rate of less than 1°C/min. Absorbances were monitored continously at 550 nm against a buffer blank, using a Cary spectrophotometer with a thermostatted cell housing. Heating and cooling rates were 0.2°C/min. In both cases temperature was measured by a thermocouple dipping into the cuvette just above the light path.

Results

The cooperativity of the phase transition in extended multibilayers and small, single-bilayer vesicles of both dimyristoyl and of dipalmitoyl phosphatidyl-



Scheme I

choline was investigated using three different spin labels I, II (12,3), III (12,3) and also using 90° light scattering and optical turbidity measurements. The results of the spin label measurement of the phase transition are given in Fig. 1. In all cases it is seen that the spectral changes at the phase transition take place over a much broader temperature range in the small, single-bilayer vesicles than the sharp transition which is observed with extended, multibilayer dispersions, i.e. the cooperativity of the transition is much higher in the latter case. TEMPO I is expected to be the more reliable indicator of the transition cooperativity, since it offers least perturbation both because of its small size and because it dissolves only in the fluid lipid, where the available free volume is greater. For this reason the TEMPO results will be concentrated on in the following, but the significant result is that qualitatively the same large difference between extended multibilayers and small, single-bilayer vesicles is observed with all three different labels.

The results on the 90° light scattering are given in Fig. 2. The reason for the anomalous increase in scattering for the dipalmitoyl phosphatidylcholine dispersion is not known, but in the absence of salt and buffer a sharp decrease is obtained and all other systems behave normally. From Fig. 2 it is seen that there is a sharp change in light scattering intensity at the phase transition of the extended multibilayer dispersions, a somewhat smaller and broader change after sonication of the dispersion, and a yet smaller, broader change after centrifugation of the sonicated dispersion. These relative changes were always in the same progression and in some cases it was found that almost no change at all was obtained in the scattering intensity of the centrifuged, sonicated dispersion, although phosphate analysis showed that less than 10% of the lipid was lost on centrifugation.

The absorbance measurements give essentially similar results to the 90° light scattering changes of Fig. 2. The uncentrifuged and the centrifuged sonicated dispersions show much smaller changes and have very much lower absolute absorbances than the unsonicated dispersions of the same concentration. Again changes at the phase transition in centrifuged, sonicated dispersions are up to a factor of 3 or more smaller than those of the uncentrifuged, sonicated dispersions, although less than 10% of the lipid is removed on centrifugation. Thus the absorbance and 90° light scattering changes of the uncentrifuged, sonicated dispersions appear to be largely accounted for by a relatively small

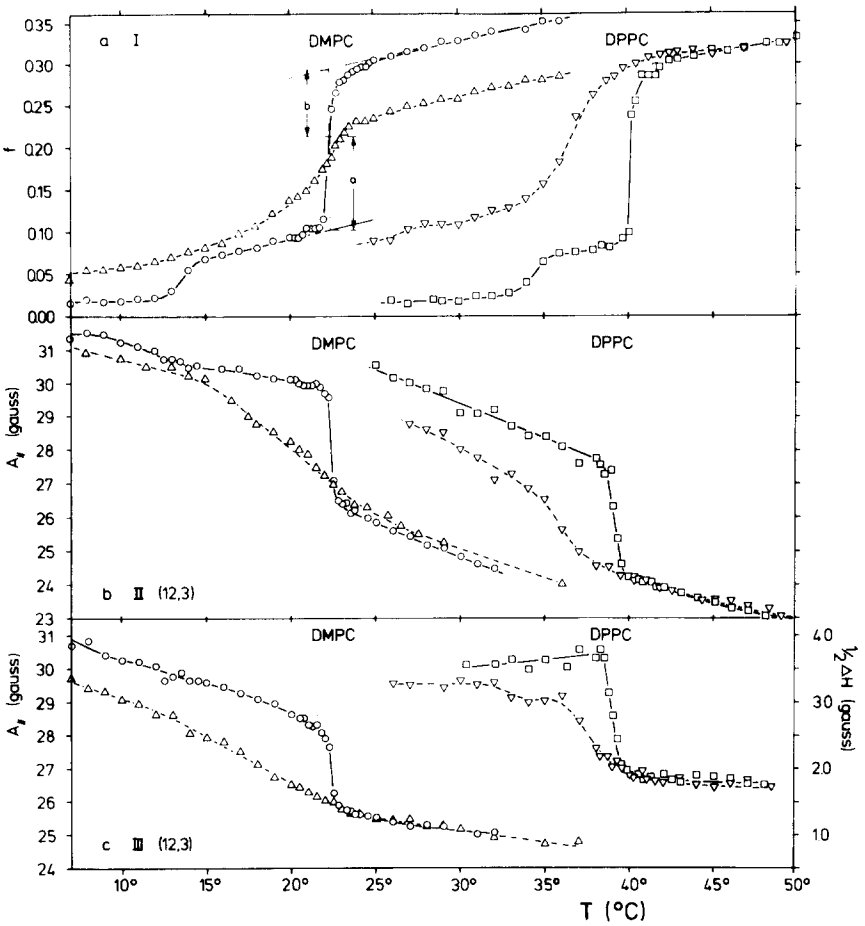


Fig. 1. ESR indications of the lipid phase transition in dimyristoyl phosphatidylcholine (DMPC) (○,△) and dipalmitoyl phosphatidylcholine (DPPC) (□,▽), multibilayer dispersions (full line) and single-bilayer vesicles (broken line). (a) TEMPO spin label I, water-lipid partition parameter (the vertical scale has been reduced by a factor 2 X for dimyristoyl phosphatidylcholine dispersion). (b) Phosphatidylcholine spin label II (12,3) A// outer hyperfine splitting. (c) Stearic acid spin label III (12,3) A// outer hyperfine splitting (dimyristoyl phosphatidylcholine) and low field line width (dipalmitoyl phosphatidylcholine). For details of spectral interpretation see refs. 4, 20, 21 and 24.

contamination of the sonicate by larger vesicles which can be mostly removed by centrifugation.

A recent theoretical analysis [45] has shown that a sonicated dipalmitoyl phosphatidylcholine dispersion, at a concentration of 1 mg/ml, which has an absorbance of less than 0.1 at 436 nm, must be composed solely of small vesicles with a narrow distribution of sizes, the mean diameter being less than 400 Å. Any appreciably higher absorbance is indicative of polydispersity in the system [45]. Applying these results to our measured absorbances confirms that the centrifuged sonicates are composed almost entirely of small, single-bilayer vesicles, and that the uncentrifuged sonicates are contaminated by larger vesicles of diameter considerably greater than 400 Å. Since these results demonstrate that the light scattering and absorbance changes at the phase

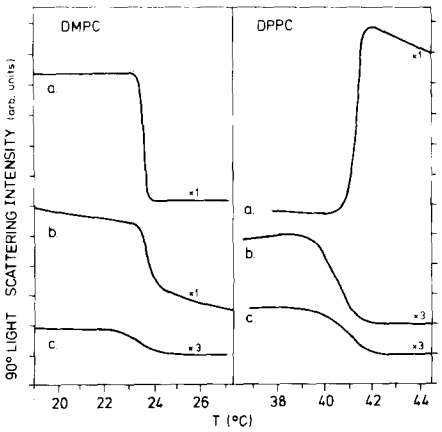


Fig. 2. 90° light scattering indication of the lipid phase transition in dimyristoyl phosphatidylcholine (DMPC) (left-hand curves) and dipalmitoyl phosphatidylcholine (DPPC) (right-hand curves) bilayers. Upper curves a, multibilayer dispersions; middle curves b, sonicated dispersions; Lower curves c, sonicated and centrifuged dispersions. (Note different gain settings).

transition of sonicated dispersions are dominated by contamination by larger vesicles, it seems that the optical measurements cannot be taken as a reliable indicator of the cooperativity in single-bilayer vesicles. Clearly the spin label measurements are much more reliable in this respect, since they will give results directly proportional to the amounts of lipid involved with no bias towards larger vesicles. An additional factor is that the optical measurements will be unduly sensitive to any small degree of vesicle fusion since this would produce larger vesicles, whereas the spin label results would be affected in direct proportion to the extent of fusion.

Theory

The Zimm and Bragg theory of cooperative transitions [22] is applied assuming there are three lipid states during the transition: the ordered state, s, the fluid state, f, and the intermediate or interfacial state, i. [6]. Two parameters are required: (i) the statistical weight of a fluid state molecule:

$$s = \exp(-F_f/RT) \tag{1}$$

where fluid state free energy is related to the transition enthalpy, ΔH_t, and entropy, ΔS_t by:

$$F_f = \Delta H_t - T\Delta S_t \tag{2},$$

and (ii) the cooperativity parameter:

$$\sigma = \exp(-F_i/RT) \tag{3}$$

where F_i is the additional free energy of the interfacial region. Cooperativity arises because F_i tends to decrease the length of the interfacial regions [6].

The degree of transition, i.e. mean fraction of molecules in the fluid state, is

[6,22,49]:

$$\theta = \tfrac{1}{2}\left[1 + \frac{s-1}{\sqrt{(s-1)^2 + 4\sigma s}}\right] \tag{4}$$

The calculated transition curves in Fig. 3a are symmetric in contrast with the experimental results of Fig. 1 which are differentially broadened towards lower temperatures. The reasons for this difference are 2-fold: (i) the calibration between s and T is not linear (Eqn. 1), (ii) residual pre-transitional effects are observed in the low temperature region of the main transition. Because of this only the central regions of the curves are used in determining σ (see below). The mean fraction of molecules in the interfacial regions is [6,23]:

$$\eta = \frac{2\sigma s}{(1+s)\sqrt{(1-s)^2 + 4\sigma s} + (1-s)^2 + 4\sigma s} \tag{5}$$

An essential quantity for the cooperativity analysis is the mean size of the regions of ordered or fluid lipid which exist at various stages throughout the transition. If the size of a region of given type (ordered or fluid) is expressed as the mean number of molecules $\langle \upsilon \rangle$, per interfacial molecule, then for fluid regions:

$$\langle \nu_f \rangle = \frac{\theta}{\eta} = \frac{1 + s + \sqrt{(1-s)^2 + 4s\sigma}}{1 - s + \sqrt{(1-s)^2 + 4s\sigma}} \tag{6}$$

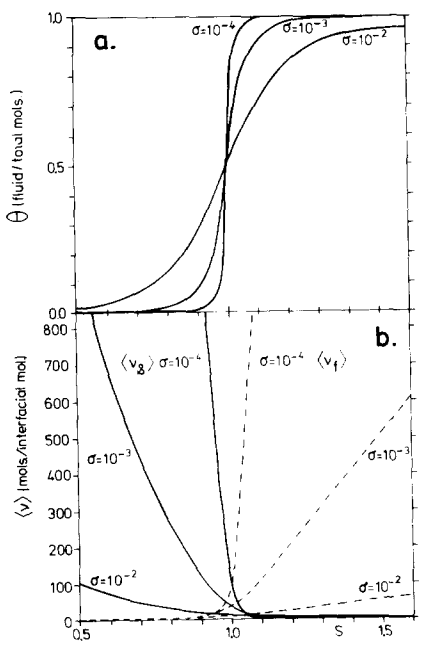


Fig. 3. Model for the cooperative transition. (a) Fraction of molecules in the fluid phase (degree of transition). (b) Size of the regions of ordered, s, and fluid, f, phase lipid (number of molecules per perimeter molecule) plotted against s; for various values of the cooperativity parameter, σ.

and for ordered regions:

$$\langle \nu_s \rangle = \frac{1-\theta}{\eta} = \frac{1+s+\sqrt{(1-s)^2+4s\sigma}}{-1+s+\sqrt{(1-s)^2+4s\sigma}} \tag{7}$$

Fig. 3b shows that for high cooperativity (low σ) the fluid regions increase very rapidly with concomitant rapid decrease in ordered regions above the transition and vice-versa below, whereas for lower cooperativity co-existence of fluid and ordered lipid, i.e. lateral phase separation [24], is possible over an appreciable range.

At the centre of the transition ($s = 1$) the mean sizes of the ordered and fluid regions are equal and are directly related to the cooperativity by:

$$\langle \nu_s \rangle_{T_t} = \langle \nu_f \rangle_{T_t} = \frac{1}{\sqrt{\sigma}} + 1 \tag{8}$$

For comparison with experiment the s dependence of Fig. 3 can be linearized in temperature by a Taylor Series expansion of Eqn. 1 about the transition temperature, T_t:

$$s \simeq 1 + \frac{\Delta H_t}{R T_t^2}(T - T_t) \tag{9}$$

Differentiating Eqn. 4:

$$\left.\frac{d\theta}{dT}\right|_{T_t} = \frac{1}{4\sqrt{\sigma}} \cdot \frac{\Delta H_t}{RT^2} \tag{10}$$

shows that θ will have a linear dependence on $1/T$ around T_t with a negative slope from which σ may be determined. The values of θ calculated from the experimental data are plotted against reciprocal temperature in Fig. 4. The values of cooperativity parameter, σ, calculated from the gradients of these plots are given in Table I.

If the ordered-fluid transition is considered as a pseudo-unimolecular reaction with equilibrium constant $K_t = \theta/(1-\theta)$, then Eqn. 10 can be transformed into the van 't Hoff form with the result that the effective reaction enthalpy can be given by: $\Delta H_{vH} = (1/\sqrt{\sigma})\,\Delta H_t$ (see refs. 12, 13 and 49). Thus the temperature dependence of the degree of transition is equivalent to a transition involving $1/\sqrt{\sigma}$ molecules simultaneously. The quantity $1/\sqrt{\sigma}$ is frequently referred to as the size of the cooperative unit and is directly related to the mean size of the ordered or fluid region at the centre of the transition, given in the present work. For large values of $1/\sqrt{\sigma}$, Eqn. 8 shows that the size of the cooperative unit is equal to the value of $\langle \upsilon \rangle$ at the centre of the transition, but it should be emphasized that $\langle \upsilon \rangle$ is the mean number of molecules per interfacial molecule in an ordered or fluid region at the mid-point of the transition, and not the total size of the particular ordered or fluid areas.

The effect of membrane morphology on the pattern of ordered and fluid domains in the membrane can be investigated using Eqns. 6—8 via the effects on the cooperativity parameter. Since $\langle \upsilon \rangle$ is the mean value of the ratio of the number of molecules in any particular ordered or fluid region to the number

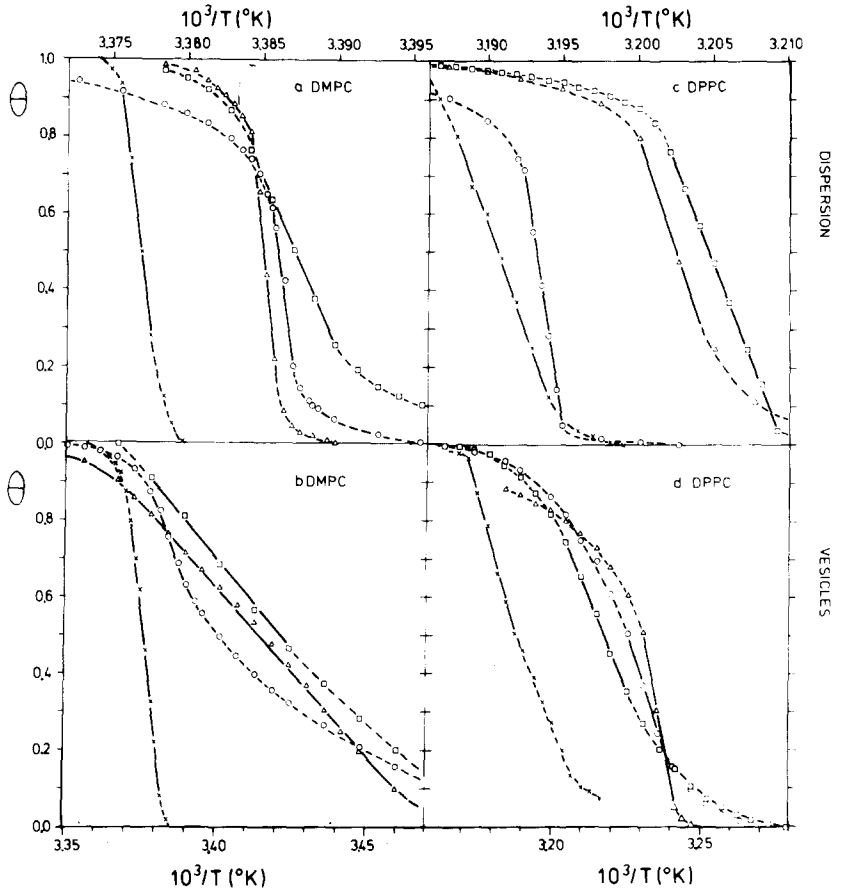


Fig. 4. Degree of transition, θ, for the lipid phase transition in phosphatidylcholine bilayers against reciprocal temperature. Upper curves: multibilayer dispersions; lower curves: single-bilayer vesicles. (N.B., the reciprocal temperature scales for the multibilayer dispersions are expanded five times compared with those for the single-bilayer vesicles). a, b, dimyristoyl phosphatidylcholine; c, d, dipalmitoyl phosphatidylcholine. Values deduced from the data for: TEMPO spin label I (○); phosphatidylcholine spin label II (12,3) (△); stearic acid spin label III (12,3) (□); 90° light scattering (X). Values for θ are obtained from the experiment data according to: $\theta = a/(a + b)$; see Fig. 1.

of molecules at the perimeter, then in general:

$$\langle \nu \rangle = \left(\frac{d}{f}\right) \cdot \left\langle \frac{A}{p} \right\rangle \tag{11}$$

where A is the area of the region, p is the length of its perimeter, f is the area per molecule, and d is the breadth of a molecule at the perimeter. In particular, for circular regions (which is the shape which would tend to minimize the total interfacial energy):

$$\langle \nu \rangle_{\text{circle}} = \left(\frac{d}{f}\right) \cdot \frac{\langle r \rangle}{2} \tag{12}$$

where $\langle r \rangle$ is the mean radius of the circular region. The way in which the pattern of lateral phase separation varies throughout the phase transition of

TABLE I

COOPERATIVITY PARAMETER OF THE LIPID PHASE TRANSITION IN DIMYRISTOYL AND DIPALMITOYL PHOSPHATIDYLCHOLINE MULTIBILAYER DISPERSIONS AND SINGLE-BILAYER VESICLES

Deduced from the various indicators of the bilayer phase transition. Values for the enthalpy of transition in dispersions are taken from refs. 25 and 26. Values for vesicles are taken to be the same as for multibilayers [27], or for dipalmitoyl phosphatidylcholine (b) corrected according to ref. 46. Values given in parentheses are the size of the cooperative unit.

	Dimyristoyl phosphatidylcholine		Dipalmitoyl phosphatidylcholine		
	Dispersion	Vesicle	Dispersion	Vesicle	
				a	b
I	$4.9 \cdot 10^{-6}$ (450)	$2.4 \cdot 10^{-3}$ (21)	$1.7 \cdot 10^{-5}$ (245)	$2.1 \cdot 10^{-3}$ (23)	$1.2 \cdot 10^{-3}$ (30)
II (12,3)	$4.6 \cdot 10^{-6}$ (465)	$5.6 \cdot 10^{-3}$ (14)	$1.3 \cdot 10^{-4}$ (89)	$8.9 \cdot 10^{-4}$ (35)	$5.1 \cdot 10^{-4}$ (46)
III (12,3)	$7.7 \cdot 10^{-5}$ (115)	$6.5 \cdot 10^{-3}$ (13)	$1.3 \cdot 10^{-4}$ (89)	$3.6 \cdot 10^{-3}$ (18)	$2.1 \cdot 10^{-3}$ (24)
L.S. (i)	$6.9 \cdot 10^{-6}$ (380)	$2.5 \cdot 10^{-4}$ (64)	$7.3 \cdot 10^{-5}$ (117)	$1.5 \cdot 10^{-3}$ (27)	$8.6 \cdot 10^{-4}$ (36)
(ii)	$5.0 \cdot 10^{-6}$ (445)	$4.7 \cdot 10^{-5}$ (145)	$1.2 \cdot 10^{-4}$ (91)	$2.4 \cdot 10^{-4}$ (66)	$1.4 \cdot 10^{-4}$ (87)
A	$2.4 \cdot 10^{-6}$ (640)	$3.1 \cdot 10^{-6}$ (596)	$1.3 \cdot 10^{-5}$ (278)	$6.1 \cdot 10^{-5}$ (128)	$3.5 \cdot 10^{-5}$ (169)

L.S., 90° light scattering: i, with salt; ii, without salt. *A*, absorbance. Values given in vesicle column are for uncentrifuged sonicates (see text).

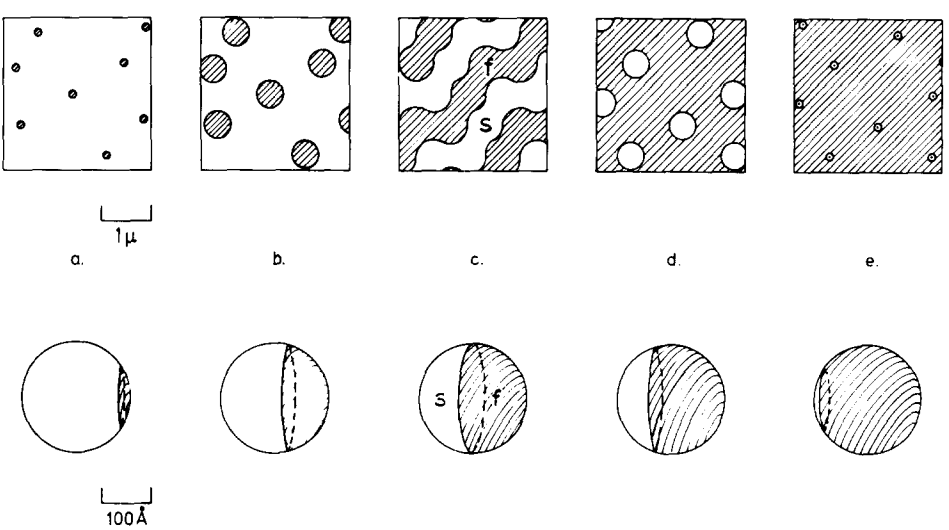


Fig. 5. Illustrative scale diagram of the lateral phase separation pattern of ordered, s, and fluid, f, domains throughout the phase transition in dimyristoyl phosphatidylcholine bilayers. Upper row: multibilayer dispersion, $\sigma = 5 \cdot 10^{-6}$: a, 0.5°C below the transition centre T_t; b, 0.1°C below T_t; c, at the centre of the transition T_t; d, 0.1°C above T_t; e, 0.5°C above T_t. Lower row: single-bilayer vesicle, $\sigma = 2.5 \cdot 10^{-3}$: a, 5°C below T_t; b, 1°C below T_t; c, at T_t; d, 1°C above T_t; e, 5°C above T_t.

TABLE II

CALCULATED VALUES OF THE MEAN SIZE OF THE ORDERED AND FLUID REGIONS AT THE CENTRE OF THE PHASE TRANSITION IN DIMYRISTOYL PHOSPHATIDYLCHOLINE VESICLES

Calculation of the area/perimeter ratio for (i) $\sigma = 2.4 \cdot 10^{-3}$, (ii) $\sigma = 5.6 \cdot 10^{-3}$ and various values of the mean molecular area, f, and molecular width, d.

f (Å^2)	d (Å)	f/d (Å)	A/p (Å)	
			i	ii
40	9.2	4.35	93	62
60	9.2	6.52	140	94
40	6.2	6.45	138	93
60	6.2	9.67	207	139

dimyristoyl phosphatidylcholine multibilayer dispersions is calculated out diagrammatically in Fig. 5 (upper row), assuming circular regions, taking the value of $\sigma = 5 \cdot 10^{-6}$ from Table I and a value of $f/d = 6.5$ (see Table II).

When dealing with small, single-bilayer vesicles the morphology of the vesicle must be taken explicitly into account. Consider, in particular, the situation at the centre of the transition in which the mean sizes of the fluid and ordered regions are equal (cf. Fig. 3 and Eqn. 8). If, in this situation, the cooperativity of the transition is limited entirely by the size of the vesicle, then the area of an ordered-fluid lipid region would be just one half of the total surface area of the vesicle (as depicted in Fig. 5c, lower row). The condition for this is that the area/perimeter ratio is $A/p = R_m$, where R_m is the mean radius of the vesicle, and from Eqns. 8 and 11:

$$\langle \nu \rangle_{ves} = \frac{1}{\sqrt{\sigma}} + 1 = \left(\frac{d}{f}\right) R_m \tag{13}$$

This possibility is tested in Table II, where values of the A/p ratios are calculated from Eqns. 8 and 11 using the experimental values of $\sigma = 2.4 \cdot 10^{-3}$ and $\sigma = 5.6 \cdot 10^{-3}$ for dimyristoyl phosphatidylcholine vesicles from Table I, and various possible combinations of f and d which span the likely range of these values. The values taken for f are 40 and 60 Å^2, being the limits for completely ordered or completely fluid bilayers, respectively (see refs. 28 and 29) and for d are 6.2 and 9.2 Å, being the limiting size of the headgroup and the maximum breadth of the two chains of the phosphatidylcholine molecule, respectively [30]. The values of A/p in Table II are to be compared with the mean radius obtained for dimyristoyl phosphatidylcholine vesicles of approx. 90 Å, and outer radius of 105 Å*. (These data were obtained by diffusion coefficient,

* At this small radius of curvature the majority of the lipid (≥70%) is present in the outer monolayer of the vesicle. Since the contribution to the degree of transition curves in Fig. 4 is directly proportional to the amount of lipid involved, it is clear that the lipid in the outer monolayer will dominate the phase transition behaviour in the centre of the transition. Calculations allowing for the effect of the different radii of curvature of the outer and inner monolayers on the cooperativity show that the inner monolayer contributes only 10—15% to the slope of the degree of transition curves in the region at the centre of the transition. As the cooperativity parameters in Table I were derived solely from the centre regions of the transition in the θ vs. T plots of Fig. 4, one can be confident that these relate essentially to the properties of the outer monolayer with very little distortion by contributions from the inner monolayer. In contrast, it is clear that in the extreme wings of the transition this would not be the case.

sedimentation velocity, partial specific volume, intrinsic viscosity, internal volume and freeze-etch electron microscopy measurements, all of which give consistent values for the mean vesicle radius: Watts, A., Marsh, D. and Knowles, P.F., to be published, and see also ref. 6). Clearly if the A/p ratio is equal to (or greater than) R_m, then the observed cooperativity is the maximum that can be expected for a vesicle of this size. It is seen that this is the case in Table II even for the smaller value of σ, implying that the cooperativity of the transition is limited by the physical size of the vesicle, in contrast to the much higher cooperativity in the relatively unrestricted multilamellar dispersions (size approx. 0.5—5 μm). On the basis of a similar calculation the evolution of the phase separation pattern throughout the transition of dimyristoyl phosphatidylcholine vesicles is depicted diagrammatically in Fig. 5 (lower row). This indicates that at all stages throughout the transition the cooperativity is limited by the vesicle size.

Discussion

The spin label results of Fig. 1 and the optical results of Fig. 2 all indicate a clear difference between the phase transition behaviour of the multilamellar lipid dispersions and the smaller, single-bilayer vesicles. This difference cannot be attributed to chemical degradation since chemical analysis of the extracted lipid after sonication revealed no such breakdown. It was argued above, that the optical results give a less reliable indication of the phase transition behaviour of the single-bilayer vesicles, because of the sensitivity to contamination by intermediate-size particles. This probably accounts for some of the sensitivity of 90° light scattering as an indicator of the phase transition [32,33] and also to the reported similarity between the optical turbidity properties of sonicated and unsonicated dispersions [34].

The finding that the phase transition is broader in single bilayer vesicles than in unsonicated dispersions is in accordance with previous dilatometric [35—37], ^{13}C NMR [38], ESR [41], calorimetric [39], laser Raman [50] and fluorescence measurements [40,46]. Reference to Eqn. 10 shows that the increased width of the transition in the single-bilayer vesicles could arise either from a decrease in the cooperativity of the transition (increase in σ) or from a decrease in the enthalpy of transition, ΔH_t. However, the calorimetric results (see refs 27 and 46) have shown that the heat of transition for single-bilayer vesicles is not very different from that obtained for multilamellar dispersions [25,26] (cf. columns a and b for dipalmitoyl phosphatidylcholine vesicles in Table I). Thus the broadening of the transition found in the present work must be attributed to a decrease in the cooperativity of the transition.

The sizes of the cooperative unit given in Table I for multilamellar dispersions are of the same order or greater than those reported calorimetrically [26], indicating that the TEMPO spin label I does not appreciably perturb the cooperativity of the phase transition. In cases for which not such a large difference has been reported between unsonicated and sonicated systems, the transition widths of the extended multilamellae are broader than in the present study, whereas those of small vesicles are comparable. This is particularly the case in a calorimetric study [27] which reported transition widths of 3.5—4°

for both multilamellae and vesicles (corresponding to $\sigma \approx 4 \cdot 10^{-3}$ for dimyristoyl phosphatidylcholine and $\sigma \approx 6.5 \cdot 10^{-3}$ for dipalmitoyl phosphatidylcholine). These widths (and cooperativities) are comparable to the present TEMPO results on vesicles but are much broader than the TEMPO transition widths in multilamellae and the corresponding calorimetric results of Hinz and Sturtevant [26].

The decreased cooperativity of the transition in single-bilayer vesicles could arise from two possible sources: from the effect of the small size of the vesicle limiting the maximum size of the cooperative unit which can undergo the transition, or from the effect of the tight radius of curvature of the lipid vesicle in disrupting the close-packing of the lipid molecules [35,41] and hence limiting the range of the cooperative interaction. The calculations given above for dimyristoyl phosphatidylcholine vesicles, strongly suggest that it is the physical size of the vesicle which limits the cooperativity of the transition, since this effect in itself is sufficient to give rise to the observed reduction in cooperativity. In addition the results with the phosphatidylcholine spin label II (12,3) in Fig. 1, indicate little difference in the lipid chain packing between sonicated vesicles and multilamellar dispersions in the fluid state, and the similarity of the spectra below the phase transition suggest that the differences in the ordered state are not very large either.

In the case of dipalmitoyl phosphatidylcholine, the vesicles are a little larger with mean radius ≈130 Å (Watts, A., Marsh, D. and Knowles, P.F., to be published) and thus if the cooperativity is limited by the vesicle size one would expect the cooperative unit to be larger than for dimyristoyl phosphatidylcholine in the ratio of the vesicle radii (Eqn. 13) i.e. by a factor of 1.5. Comparing the values in Table I it is clear that this may be the case, but the uncertainties in calorimetric enthalpy (columns a and b for dipalmitoyl phosphatidylcholine vesicles) make it impossible to be certain on this point. The fact that the dipalmitoyl phosphatidylcholine vesicle transition temperature is depressed by 2—5°C relative to the unsonicated dispersions suggests that a slight disruption of the chain packing in the ordered-state vesicle could also contribute to the reduction in cooperativity. Again the results with spin label II (12,3) indicate little difference in chain packing between dipalmitoyl phosphatidylcholine vesicles and unsonicated dispersions above the phase transition.

It is thus clear that membrane morphology is capable of quite drastically affecting the cooperativity of lipid bilayer phase transitions and that in the case of small, single-bilayer phospholipid vesicles the physical effects of the tight radius of curvature of the vesicle on molecular packing may also be important. Reference to Fig. 5c (upper row) suggests that the lateral phase separation pattern in extended dimyristoyl phsophatidylcholine dispersions may also be limited by vesicle size if this is in the region of 1 μm diameter. (Calculation for spherical vesicles with $\sigma = 5 \cdot 10^{-6}$ shows that the critical diameter is 0.6 μm). Such effects are apparently seen in freeze-etch electron microscopy of phosphatidylcholines quenched from within a lateral phase separation region [43], when the pattern of ordered and fluid lipid seems to consist of large, single domains. Thus it is possible that the situation of extended bilayer dispersions approaches more closely that depicted in the lower row of Fig. 5 than that in the upper row, and the cooperativity, though much greater, may nevertheless

be limited by the vesicle size. A further feature of the freeze-etch studies which supports this conclusion is the fact that the phase boundaries in saturated phosphatidylcholines are straight rather than curved or circular (Kleeman, W., private communication and ref. 43). Curved or circular boundaries have been observed in systems containing phosphatidylethanolamines and/or unsaturated fatty acid chains [42,48], and it is possible that the model of Fig. 5 (upper row) may be more applicable to these systems.

The mean size of the ordered or fluid regions is relevant to the possible realization of lateral communication within the plane of the membrane, i.e. the simultaneous transition at a given point on the membrane surface arising from the localized triggering of the transition at a different point, or a simultaneous transition at two different points arising from a non-localized triggering of the transition. It is evident from the discussion above and from Fig. 5 (lower row) that lateral communication can be achieved across the entire periphery of a 200 Å dimyristoyl phosphatidylcholine vesicle, since the ordered or fluid domain extends over the entire surface. In the case of the dimyristoyl phosphatidylcholine multilamellar dispersions the cooperativity is much higher and the communication can be much longer range. Taking as an index for the range of cooperativity the mean radius of notional circular domains of fluid or ordered lipid at the centre of the transition *, we get lateral communication over distances of the order 0.6 μm for a cooperative unit size of 460 molecules corresponding to unsonicated dimyristoyl phosphatidylcholine. Such highly cooperative transitions are not observed in natural membranes, but lateral communication may be possible in localized regions of the membrane, e.g. between the components of the cytochrome *P*-450 reductase system [15], or between the cytochrome and reductase elements of the cytochrome b_5 reductase system [51] in liver microsomes. Long range lateral communication has also been demonstrated in reconstituted lipid-protein systems where freeze-etch electron microscopy reveals that integral membrane proteins are completely excluded from the ordered lipid domains [42,43].

In conclusion it should be emphasized that, since the cooperative unit corresponds to the mean number of molecules in a given ordered or fluid domain per perimeter molecule, in principle quite broad transitions can give rise to distinct solid or fluid domains of appreciable size. Thus even the very broad transitions observed in natural membranes could be responsible for lateral communication over functionally significant distances. For instance, a transition which exhibits a width of 20°, corresponding to a cooperative unit size of three lipid molecules, could in principle give rise to lateral communication over a distance of about 50 Å.

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* It can be shown that for a change in s over the range $1 \pm \sqrt{\sigma}$ the resulting change in $\langle v \rangle$ is $1/\sqrt{\sigma}$.

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