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Evidence for Phase Boundary Lipid. Permeability of Tempo-choline into Dimyristoylphosphatidylcholine Vesicles at the Phase Transition[†]

Derek Marsh,* Anthony Watts,[‡] and Peter F. Knowles

ABSTRACT: The existence of distinct regions of mismatch in molecular packing at the interfaces of the fluid and ordered domains during the phase transition of dimyristoylphosphatidylcholine vesicles has been demonstrated by measuring the temperature dependence of the permeability to a spin-label cation and comparing this with a statistical mechanical calculation of the fraction of interfacial lipid. The kinetics of uptake and release of the 2,2,6,6-tetramethylpiperidinyl-1-oxycholine (Tempo-choline) spin label by single-bilayer dimyristoylphosphatidylcholine vesicles were measured using electron spin resonance spectroscopy to quantitate the amount of spin label present within the vesicles after removal of the external spin-label by ascorbate at 0 °C. Both the uptake and release experiments show that the Tempo-choline permeability peaks to a sharp maximum at the lipid-phase transition, the vesicles being almost impermeable to Tempo-choline below the transition and having a much reduced permeability above. The temperature profile of the permeability is in reasonable quantitative agreement with calculations of the fraction of interfacial boundary lipid from the Zimm and Bragg theory of cooperative transitions, which use independent spin-label measurements of the degree of transition to determine the cooperativity parameter. The relatively high intrinsic permeability of the interfacial regions ($P \sim 0.2-1.0 \times 10^{-8}$ cm/s) is attributed to the mismatch in molecular packing of the lipid molecules at the ordered-fluid boundaries, which could have important implications not only for permeability in natural membranes (e.g., in transmitter release), but also for the function of membrane-bound enzymes and transport proteins.

The possible functional importance of the lipid-phase transition and of the conditions of lateral lipid-phase separation is now well established, as a result of many studies on both biological and model membranes (see e.g., Marsh, 1975).

The increase in fluidity at the phase transition has been shown to enhance the activity of enzymes and specific transport systems within the membrane (Overath and Träuble, 1973; Esfahani et al., 1972) and the possible physiological significance of lipid-phase transitions in triggering these membrane functions has been emphasized (Träuble, 1972; Träuble and Eibl, 1973). Lateral phase separation of lipids within *Escherichia coli* plasma membranes has also been shown to have a dramatic effect on sugar transport by the membrane (Linden et al., 1973).

These effects can be attributed to the more expanded and more flexible nature of the lipid chains in the fluid state relative to the ordered state of the chains below the phase transition (Hubbell and McConnell, 1971; Marsh, 1974a). Conformational changes of embedded proteins are thus facilitated in the fluid state above the phase transition, and transitions from the expanded to the condensed state give rise to a high lateral compressibility of membranes in a state of lateral phase separation. This in turn also facilitates conformational transitions of membrane proteins and could also accommodate the introduction of newly synthesized proteins and lipids into the membrane (Linden et al., 1973).

Of possibly equal importance are the properties of the

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membrane lipid that is actually undergoing a phase transition, or more generally of the interfacial lipid in the boundary regions which exist between fluid and ordered phases of membranes in a condition of physical lateral phase separation. Because of the mismatch in molecular packing between the ordered and fluid lipid, these interfacial regions could have important, possibly even catastrophic, effects on membrane function, as suggested by Papahadjopoulos et al. (1973).

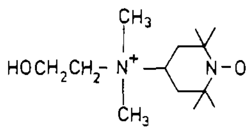
In the present paper we report experiments that give evidence for the existence of such interfacial lipid in the membrane of DMPC[1] vesicles. This phase boundary lipid is shown to be the determining factor for the cation permeability of the vesicles at the phase transition. In these experiments the permeability of single-bilayer vesicles to the spin-label cation, Tempo-choline, was measured as a function of temperature, throughout the phase transition. The measurements were made using ESR spectroscopy to quantitate the amount of spin-label present within the vesicles, after removal of the external spin-label with ascorbate at 0 °C. The correlation of the permeability with the phase boundary regions was established using calculations based on the Zimm and Bragg theory of cooperative transitions.

Experimental Section

Materials. DMPC was obtained from Calbiochem (chromatographically pure, grade I, various lot numbers) and its purity was checked by two-dimensional TLC (Hallermayer and Neupert, 1974). Square silicic acid TLC plates (Merck, Darmstadt, Germany) were run with chloroform–acetone–methanol–acetic acid–water (56:20:10:10:4) in one direction and the solvent system chloroform–methanol–25% (v/v) ammonia (65:35:5) in the second direction. The plates were first developed with "Dittmer" reagent (Dittmer and Lester, 1964) to detect the lipid phosphate and subsequently with iodine vapor to detect the total lipid. A single spot was obtained with a loading of 30 µl of 5 mg/ml of chloroform solution of DMPC and no impurities were detected with a sixfold overloading. Gas–liquid chromatography, performed by Dr. A. Sheltawy of the Biochemistry Department, Leeds University, revealed a contamination of less than 0.5% palmitic chains in the DMPC samples. Since no free fatty acids nor lipids other than PC were detected by TLC, this small impurity is attributed to dipalmitoylphosphatidylcholine.

The spin label Tempo-choline (I) was prepared in the Biophysics Department, Leeds, by Dr. R. P. Gregson according to the method of Kornberg and McConnell (1971). The spin-label Tempo (II) was prepared according to the method of Rozantzev and Neiman (1964). Spin-labeled dipalmitoylphosphatidylcholine (III) was prepared by Dr. R. P. Gregson according to the method of Hubbell and McConnell (1971).

I



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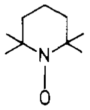

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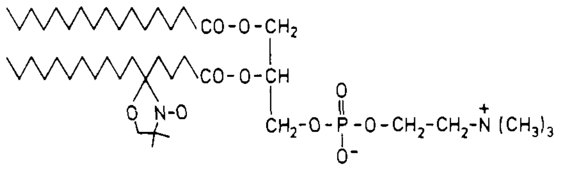

The buffer used for all measurements was 0.1 M KCl/0.01 M Tris adjusted to pH 8.0. All other reagents were of analytical grade.

Vesicle Preparation for Uptake Measurements. DMPC was suspended in 1.5 ml of buffer to a concentration of 50 mg/ml and the mixture was sonicated with a DAWE Soniprobe Type 6400 A equipped with a ⅛ in. titanium microtip. Sonication was performed in 10–25-s pulses, using the lowest power level necessary to produce sonication, until the sample became optically clear (usually 1–2 min duration). The sonication vessel was cooled with melting ice, but the temperature was allowed to rise up to 50 °C during sonication. The sample was cooled after sonication and then centrifuged at 105 000*g* at 4 °C for 10 min to remove undispersed lipid and titanium fragments. Then 1.5 ml of 6 mM Tempo-choline in buffer, previously cooled to 4 °C was added to the clear supernatant. This and all subsequent operations were carried out at 4 °C. It was demonstrated by the methods described below that the vesicles are totally impermeable to Tempo-choline at this temperature; the sonication conditions used here thus correspond to the high-temperature annealed samples of Lawaczek et al. (1975). The final lipid concentrations were checked by spectrophotometric phosphate analysis (Gomori, 1942) and the samples were all found to contain greater than 80% of the original lipid. The samples so prepared thus contained approximately 25 mg/ml of DMPC as single-bilayer vesicles (see below) and 3 mM Tempo-choline spin-label that was completely excluded from the occluded vesicle volume. This latter point was checked by ESR spectroscopy as described below.

Vesicle Preparation for Release Experiments. DMPC was suspended in 3 ml of 3 mM Tempo-choline in buffer to a lipid concentration of 25 mg/ml. Sonication was performed as described above. After cooling, the sample was then dialysed over a 24-h period against three separate changes of 3 l. of buffer, at 4 °C. After dialysis, the vesicle suspension was centrifuged at 105 000*g* at 4 °C for 10 min. The final lipid concentration of the clear supernatant was again checked by phosphate analysis. The preparation thus contained single bilayer vesicles with 3 mM Tempo-choline in the occluded volume within the vesicles and a very low concentration of Tempo-choline outside the vesicles. The latter point was checked by ESR spectroscopy as indicated below.

Permeability Measurements. Both Tempo-choline uptake and release were measured from the time course of the line height of the ESR spectrum from the Tempo-choline inside the vesicles, using the two different sets of samples as prepared above, i.e., with Tempo-choline initially outside, or inside the vesicles, respectively. All samples were maintained at 4 °C, except during incubation at the required temperature. Duplicate 50-µl aliquots of clear vesicle suspension were incubated in sealed plastic test tubes at the required temperature for given times. Duplicate control samples were similarly incubated. The samples were then cooled in melting ice, and all subsequent operations performed with samples and reagents at 0 °C. Aliquots of 10 µl of ice-cold 0.35 M sodium ascorbate were added to the samples and 10-µl aliquots of ice-cold buffer were added to the controls. The samples and controls were then transferred to 100-µl "Microcap" capillaries (Drummond, Scientific Co.) that were sealed at one end, for quantitation of the Tempo-

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[1] Abbreviations used are: DMPC, dimyristoylphosphatidylcholine; ESR, electron spin resonance; TLC, thin-layer chromatography; PC, phosphatidylcholine; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

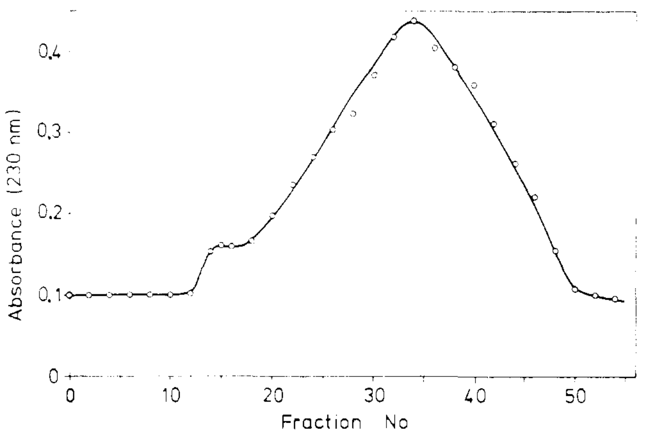

FIGURE 1: Elution profile of dimyristoylphosphatidylcholine vesicles (50 mg in 2 ml), sonicated above the phase transition temperature and then fractionated at 32 °C on Sepharose 2B.

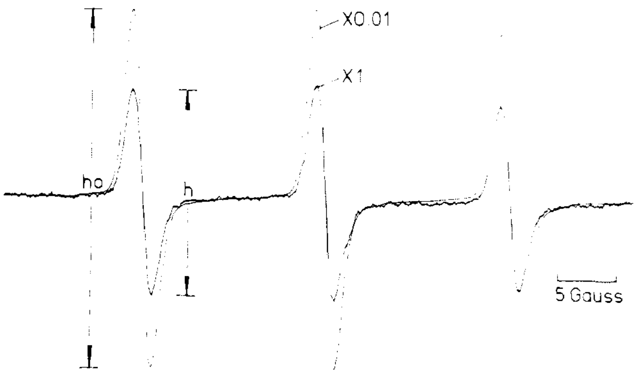

FIGURE 2: ESR spectra at 0 °C of Tempo-choline spin-label I. Heavy line (h): spectrum of Tempo-choline taken up into sonicated vesicles of dimyristoylphosphatidylcholine. Vesicles incubated for 20 min, at 29 °C with 3 mM Tempo-choline added to the outside, then treated with ascorbate at 0 °C prior to taking the spectrum. Fine line (h_0): spectrum of control sample, similarly incubated but without added ascorbate (note decreased gain).

choline content by ESR. Measurement of the ESR spectrum line height of the ascorbate-treated samples, h, relative to the controls, h_0, gives the quantity of Tempo-choline trapped in the vesicles. Hence the absolute concentration of Tempo-choline remaining in the vesicles can be calculated in terms of the original 3 mM Tempo-choline concentration in the sample.

The ESR spectra were recorded at 0 °C on a Decca X1 9 GHz ESR spectrometer, using a quartz dewar nitrogen gas flow system. The samples were lowered into and out of a standard 4-mm quartz ESR tube that was left permanently in the cavity. In this way it was possible to change samples with minimal retuning of the cavity. Only the calibrated attenuator in the detector amplifier was changed for recording the different spectra. In this way good reproducibility in the recording of signal heights was obtained. Line heights were measured of the low-field line of the nitroxide-triplet spectrum, since this spectral region is not complicated by the ascorbate free radical signal. All measured line heights were standardized relative to the appropriate control samples as explained above.

Phase Transition Measurements. The Tempo spin-label (II) and the dipalmitoylphosphatidylcholine spin-label (III) were used as indicators of the DMPC phase transition. In the former case, the DMPC was dispersed at a concentration of 25 mg/ml in a 10^{-4} M solution of Tempo in buffer. Sonication was then carried out as described for the permeability experiments. For experiments involving the dipalmitoylphosphatidylcholine spin-label, a chloroform solution of DMPC plus spin-label at a 100:1 mole ratio was evaporated under vacuum, after which the lipid was lyophilized from a minimal volume of benzene. The anhydrous lipid mixture was then dispersed at a concentration of 25 mg/ml in the standard Tris buffer, and sonicated as described for the permeability experiments. ESR spectra were recorded at 1-2 °C intervals, using heating rates of about 15 °C/h. During the 2 h required for each experiment, no change was seen in the appearance of the vesicle suspension, and on cooling the sample, no appreciable hysteresis was observed.

Column Chromatography of Vesicles. Column chromatography of DMPC vesicles, prepared as described for the permeability experiments, was performed on a Sepharose 2B column jacketed at 32 °C. The method was essentially similar to that described by Huang (1969) for the fractionation of egg lecithin vesicles. Two milliliters of 25 mg/ml of sonicated DMPC suspension in buffer was applied to the column and eluted with buffer in 3-ml fractions. The optical density of the eluant was monitored at 230 nm.

Electron Microscopy. For freeze-etching, DMPC vesicle suspension, prepared as for the permeability experiments, was placed on copper disks and rapidly frozen in liquid Freon 22. Specimens were fractured at −100 °C in a NGN680 freeze-etch apparatus, and etched by sublimation for 15 s. Freeze-etch replicas were examined with a Phillips EM 200 electron microscope operating at 60 or 80 kV.

Results

The elution profile of sonicated DMPC vesicles fractionated on Sepharose 2B is given in Figure 1. The sonication conditions used in this preparation were identical with those used in the preparation of vesicles for the permeability experiments. These results show that under these sonication conditions, the DMPC dispersion is converted almost entirely into small vesicles which are retarded by the gel (cf. Huang, 1969). Unchromatographed vesicles were used for the permeability experiments because it was found that the eluted vesicles were unstable to reconcentration (by vacuum dialysis) to the levels required for the permeability assay. The freeze-etch electron microscopy also revealed that the unfractionated, sonicated material consisted almost completely of small, single-bilayer vesicles of outside diameter approximately 200 Å.

Figure 2 shows a typical spectrum of Tempo-choline at 0 °C taken up into the occluded volume of DMPC vesicles. The spectrum from external Tempo-choline was removed by treatment with ascorbate at 0 °C, prior to recording the spectrum. The spectrum of the control sample (with no added ascorbate) is also included for comparison. The results of experiments to investigate the time course of the *uptake*, at various temperatures, of Tempo-choline added outside the vesicles are given in Figure 3. The uptake rises steeply initially, giving a measure of the permeability of the vesicles to Tempo-choline on the outside, and then flattens off to a limiting value which is a measure of the final, equilibrium uptake by the vesicles. The zero-time values for the uptake in Figure 3 are seen to be relatively small, confirming that initially the Tempo-choline is essentially excluded from the vesicles. (The zero-time ESR signal is attributed to Tempo-choline bound to the lipid and inaccessible to ascorbate, rather than being in solution within the vesicles.) It was found that this zero-time value remained constant for vesicles stored at 4 °C for periods

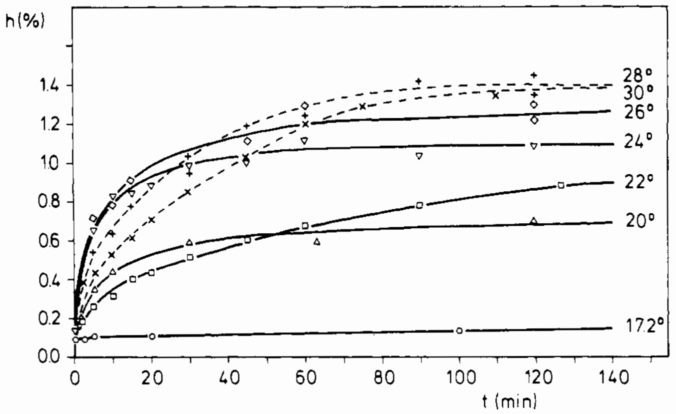

FIGURE 3: Time course of *uptake* of Tempo-choline I into dimyristoylphosphatidylcholine vesicles, on incubation at various temperatures. Plotted is the height of the Tempo-choline ESR spectrum at 0 °C (expressed as a percentage of the line-height of the 3 mM Tempo-choline originally outside the vesicles—the control) remaining after treatment of the vesicles with ascorbate at 0 °C, against the time of incubation of the vesicles. Incubation temperature: (○) 17.2 °C, (△) 20 °C, (□) 22 °C, (▽) 24 °C, (◇) 26 °C, (+) 28 °C, (×) 30 °C.

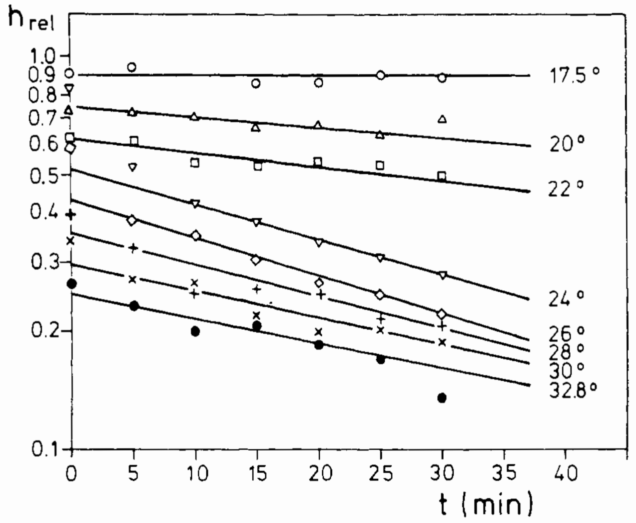

FIGURE 4: Time course of *release* of Tempo-choline I contained within dimyristoylphosphatidylcholine vesicles, on incubation at different temperatures. The plot is of the normalized Tempo-choline ESR spectrum height (logarithmic scale) at 0 °C, remaining after treatment of the vesicles with ascorbate at 0 °C, against time of incubation of the vesicles. Incubation temperature: (○) 17.5 °C, (△) 20 °C, (□) 22 °C, (▽) 24 °C, (◇) 26 °C, (+) 28 °C, (×) 30 °C, (●) 32.8 °C. For clarity, the plots at the different temperatures are displaced relative to one another.

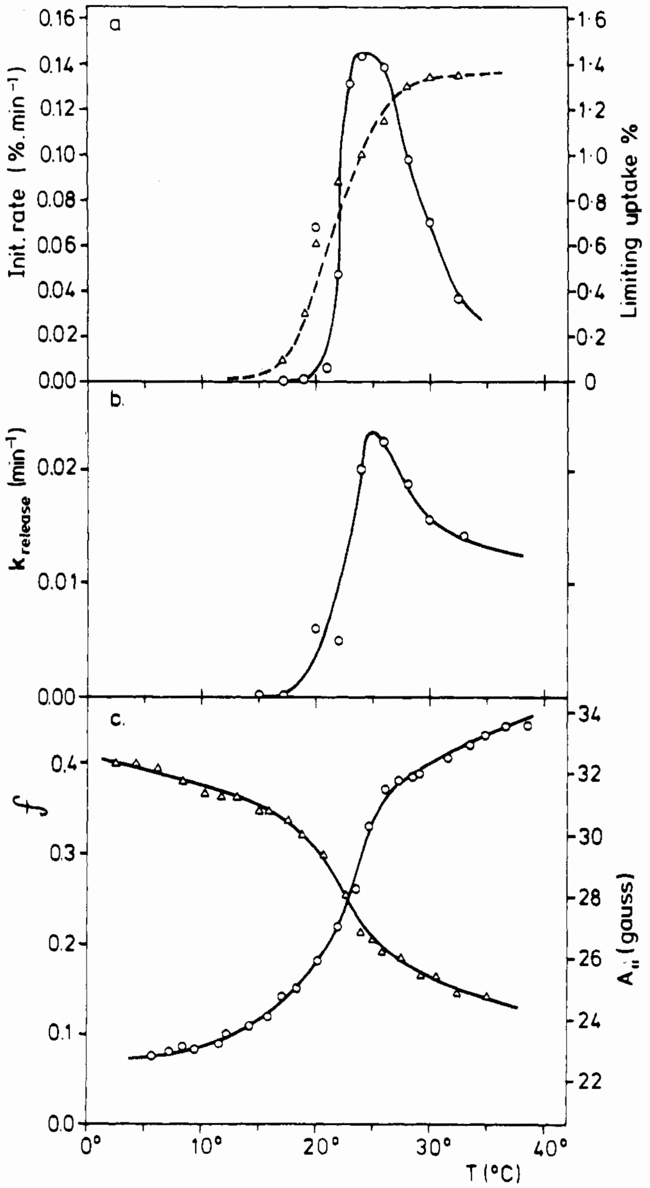

FIGURE 5: (a) Temperature dependence of the initial rate of uptake of Tempo-choline into dimyristoylphosphatidylcholine vesicles (— ○ —), and of the limiting uptake after a long period of incubation (- - △ - -). (Deduced from the data of Figure 3.) (b) Temperature dependence of the rate constant for Tempo-choline release from dimyristoylphosphatidylcholine vesicles. (Deduced from the data of Figure 4.) (c) Temperature dependence of spin-label indicators of the phase transition in dimyristoylphosphatidylcholine vesicles. (○) Fractional partition of Tempo spin-label II into the vesicles, (△) $A_\parallel$ hyperfine splitting of the dipalmitoylphosphatidylcholine spin-label III intercalated in the vesicles.

of up to 24 h, showing that the vesicles are stable for reasonably long periods under these conditions. This point is important, since samples were stored at this temperature prior to the start of incubation, and in the release experiments samples were dialyzed overnight at this temperature. Similar results on the time course of the *release* of trapped Tempo-choline from DMPC vesicles are given in Figure 4. Here a logarithmic plot of the quantity of residual Tempo-choline within the vesicles against time is used, and the first-order rate constants obtained from the slopes are a direct measure of the permeability of the vesicles to Tempo-choline. The zero-time values from the release experiment samples showed that approximately twice the signal intensity was recorded from the control sample, implying that equal quantities of Tempo-choline were present within and outside the vesicle. Since the volume within the vesicles is only about 1% of the total sample volume (as seen by the limiting uptakes in Figure 3), the initial external Tempo-choline concentration is thus only 1% of the internal concentration and contributes negligibly to the concentration gradient across the bilayer.

The temperature profiles of the vesicle permeability are compared in Figure 5 with the temperature response of spin-labels that indicate the structural lipid phase transition. Figure 5a gives the initial slope of the uptake curves in Figure 3. This is directly related to the inward permeability of the vesicles. Figure 5b gives the first-order rate constant for the Tempo-choline release deduced from Figure 4. This is directly related to the outward permeability of the vesicles. Figure 5c gives the temperature dependence of the outer splitting, $2A_\parallel$, in the ESR

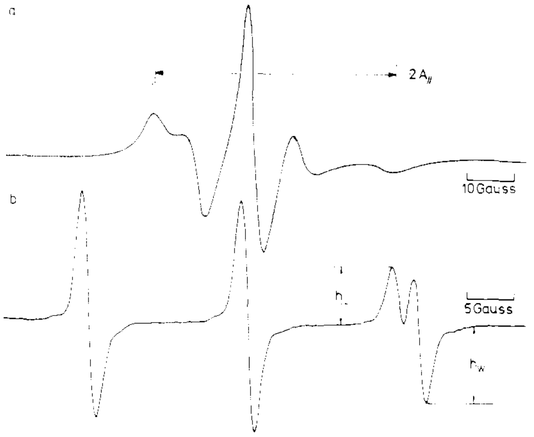

FIGURE 6: (a) ESR spectrum at 31 °C of the dipalmitoylphosphatidylcholine spin-label III in sonicated vesicles of dimyristoylphosphatidylcholine. (b) ESR spectrum at 31 °C of the Tempo spin-label II in a sonicated dispersion of dimyristoylphosphatidylcholine. h_W indicates the spectrum from II in the aqueous phase and h_L the spectrum from II partitioned into the lipid. The fractional partition is: $f = h_L/(h_W + h_L)$.

spectrum of the phospholipid spin-label III, and also the temperature dependence of the partitioning of the Tempo spin-label II into the bilayer. Representative spectra are given in Figure 6 that defines the measured parameters. The outer splitting in the spectrum of III is a measure of the amplitude of motion of the phospholipid chains, and the partitioning of II is a measure of the fluidity of the lipid membrane (see, e.g., Smith et al., 1976; Knowles et al., 1976). As can be seen from Figure 5c, both of the spin-labels are indicators of the lipid phase transition. Comparison of Figure 5a,b with 5c shows that the DMPC vesicles are completely impermeable to Tempo-choline below the phase transition, and the permeability peaks to a maximum at the center of the phase transition, becoming much lower again above the phase transition.

Since the DMPC suspension used consists of relatively homogeneous, single-bilayer vesicles it is possible to derive the permeability coefficients and effective diffusion coefficients for the transport of Tempo-choline through the DMPC bilayer. The permeability coefficient, P, is given by the following equations for the uptake and release experiments, respectively:

$$P = R_{\text{init}}\, v_0/A \tag{1}$$

$$P = k_{\text{release}}\, v_i/A \tag{2}$$

where R_{init} is the initial, *fractional* rate of uptake, k_{release} is the first-order rate constant for the release process, v_0 is the total sample volume, and $v_i = \phi \times v_0$ is the total internal volume of the vesicles, ϕ being the fractional occluded volume (ϕ can be taken as 0.014, as measured from the limiting Tempo-choline uptake in Figure 5a). The total vesicle surface area, A is given by:

$$A = a_{\text{ves}} n_{\text{ves}} \tag{3}$$

where the area per vesicle, a_{ves} and the total number of vesicles, n_{ves}, can be calculated if the mean vesicle radius, r_m (taken as measured to the center of the bilayer), and the vesicle molecular weight, M, respectively, are known. The appropriate relationships are:

$$a_{\text{ves}} = 4\pi r_m^2 \tag{4}$$

$$n_{\text{ves}} = (c_{\text{lipid}}/M) N_{\text{Av}} v_0 \tag{5}$$

where c_{lipid} is the lipid concentration in g cm^{-3} and N_{Av} is Avogadro's number. The following values for the mean vesicle dimensions have been obtained: $r_m = 89$ Å, $M = 1.9 \times 10^6$ and the bilayer thickness: $d = 31$ Å (Watts, Knowles, and Marsh, to be published). These figures yield the following values for the permeability coefficients measured at the permeability maxima in Figure 5a,b: $P = 3.0 \times 10^{-10}$ cm/s and $P = 6.8 \times 10^{-11}$ cm/s for the uptake and release experiments, respectively. The corresponding diffusion coefficients, $D = P \times d$, are: $D = 9.4 \times 10^{-17}$ cm^2/s and $D = 2.1 \times 10^{-17}$ cm^2/s for uptake and release, respectively. The difference in permeability coefficients from the uptake and release experiments can be attributed to the different time domains over which the permeabilities have been measured. In the uptake experiments the permeability was measured from the initial rate in the first 5 min, whereas in the release experiments the permeability was obtained from a rate constant measured over a 30-min period. If the rate constant for Tempo-choline release at 24 °C is measured solely over the first 5 min, then the resulting permeability coefficient is $P = 2.8 \times 10^{-10}$ cm/s in good agreement with results from the uptake experiment. The Tempo-choline permeability thus appears to be biphasic, but the important result is that the permeability peaks to a sharp maximum at the transition temperature in both the fast and the slow phases.

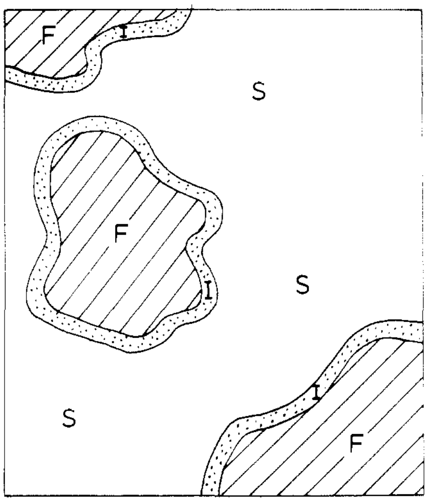

FIGURE 7: Illustration of the lateral configuration in the surface of a lipid vesicle part way through the phase transition. S are areas of ordered phase lipid molecules, F are areas of fluid phase lipid molecules, I are interfacial areas of lipid molecules which are undergoing the transition.

Theory

Model for the Cooperative Transition. In this section the Zimm and Bragg (1959) theory of cooperative helix–coil transitions is applied to the calculation of the numbers of molecules in the various states at different stages of the ordered-to-fluid transition in the phospholipid bilayer. These calculations are characterized by a cooperativity parameter that has its origin in the interfacial energy between the fluid and ordered regions of lipid and can be related to the width of the transition and the effective size of the cooperative unit in the transition.

It is assumed that there are essentially three states that the lipid molecules can take up during the transition, namely the ordered state, s, the fluid state, f, or the interfacial region between the ordered and the fluid phases, i (see Figure 7). The free energies of the molecules in these three states are determined primarily by contributions: from the internal energy of the molecules arising from rotational isomerism in the lipid chains, from the intermolecular van der Waals forces between the chains, from the electrostatic interactions between the polar headgroups, and from the configurational disorder of the lipid chains (see, e.g., Marsh, 1974b). If a molecule in the ordered, all-trans state is arbitrarily assumed to have free energy $F_s = 0$, and the free energy of a molecule in the fluid state surrounded by other molecules in the fluid state is F_f relative to the ordered state, then the free energy of a molecule at the interface between fluid and ordered regions can then be given by: $F_f + F_i$, where F_i is the additional free energy which arises from the mismatch in molecular packing between the ordered and fluid domains. (F_i is arbitrarily assigned as the additional energy of a "fluid molecule", since the interfacial energy has only to be counted once.) The mismatch in packing causes the interfacial state to be energetically unfavorable because the disruption of the attractive van der Waals energy is greater than the gain in entropy from configurational disorder. This arises from the rapid distance-dependence of van der Waals forces. F_i thus has the character of an interfacial energy that tends to reduce the number of molecules at phase boundaries, hence giving rise to the cooperativity in the system.

Statistical Mechanics. The cooperativity that arises from it being energetically more favorable for a molecule to be surrounded by molecules in the same state as itself can be summarized in a statistical weight matrix (see, e.g., Flory, 1969), whose elements $u_{\zeta\eta}$ are the statistical weights of molecules in a state ζ with the surrounding molecules in state η ($\eta = \zeta$, only if *all* the surrounding molecules are in state ζ):

$$U = [u_{\zeta\eta}] = \begin{array}{c|cc|} & \text{s} & \text{f} \\ \text{s} & 1 & \sigma s \\ \text{f} & 1 & s \end{array} \quad (6)$$

$$s = \exp(-F_f/RT) \quad (7)$$

$$\sigma = \exp(-F_i/RT) \quad (8)$$

The molecular partition function is given by:

$$Z = \sum_{n_s,n_f,n_i} z_s^{n_s} z_f^{n_f} z_i^{n_i} \quad (9)$$

where n_s and n_f are the numbers of molecules in the ordered and fluid states, respectively, and n_i is the number of fluid state molecules which are present at the interface, the total number of molecules being $n = (n_s + n_f)$. z_s and z_f are the molecular partition functions of molecules in the ordered and fluid states, respectively, and z_i is the molecular partition function corresponding to the excess energy of molecules at the interface. Substituting for the molecular partition functions, eq 9 becomes:

$$Z = \sum_{n_f=0}^{n} s^{n_f} \sigma^{n_i} \quad (10)$$

This sum may be evaluated, allowing for the proportion of molecules which are present at the interface, by sequential multiplication of the statistical weight matrix (Flory, 1969):

$$Z = (1 \quad 0)\,\mathbf{U}^n \begin{vmatrix} 1 \\ 1 \end{vmatrix} \quad (11)$$

The evaluation is facilitated by diagonalizing the statistical weight matrix; the roots of the secular equation of eq 6 are:

$$\lambda_{1,2} = \tfrac{1}{2}[(1 + s) \pm \sqrt{(1 - s)^2 + 4s\sigma}] \quad (12)$$

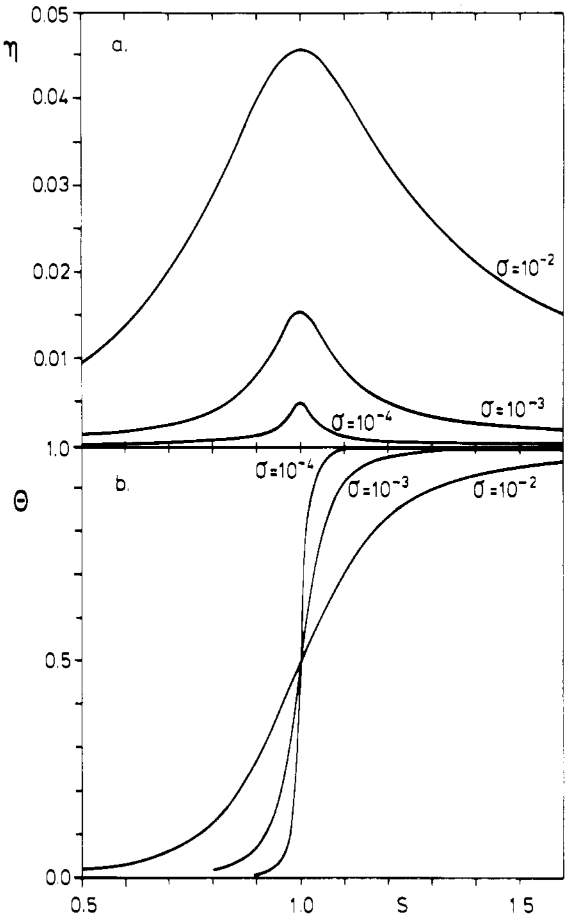

FIGURE 8: Model for the cooperative transition. (a) Fraction of molecules at the phase boundaries (interfacial molecules) plotted against the normalized temperature parameter, s. (b) Fraction of molecules in the fluid phase (degree of transition) plotted against s, for various values of the cooperativity parameter, σ.

Since the number of molecules, n, is large, the following approximation for the partition function then holds:

$$Z = \lambda_1^{\,n} \quad (13)$$

where λ_1 is the larger root.

From eq 10 and 13 it can be shown that the mean fraction of molecules, θ, in the fluid state (or the degree of transition) is:

$$\theta = \frac{\langle n_f \rangle}{n} = \frac{1}{n}\frac{\partial \ln Z}{\partial \ln s} = \frac{\partial \ln \lambda_1}{\partial \ln s} \quad (14)$$

and the mean fraction of molecules, η, in the interfacial region between the fluid and ordered phases is:

$$\eta = \frac{\langle n_i \rangle}{n} = \frac{1}{n}\frac{\partial \ln Z}{\partial \ln \sigma} = \frac{\partial \ln \lambda_1}{\partial \ln \sigma} \quad (15)$$

Substitution from eq 12 leads to the following expressions:

$$\theta = \frac{1}{2}\left[1 + \frac{s - 1}{\sqrt{(s - 1)^2 + 4\sigma s}}\right] \quad (16)$$

and

$$\eta = \frac{2\sigma s}{(1 + s)\sqrt{(1 - s)^2 + 4\sigma s} + (1 - s)^2 + 4\sigma s} \quad (17)$$

The fraction of molecules, η, in the interfacial region and the degree of transition, θ, are plotted against s for various values of σ in Figure 8. The qualitative agreement of Figure 8a,b with the temperature dependence of the Tempo-choline permeability (Figure 5a,b) and the spin-label indication of

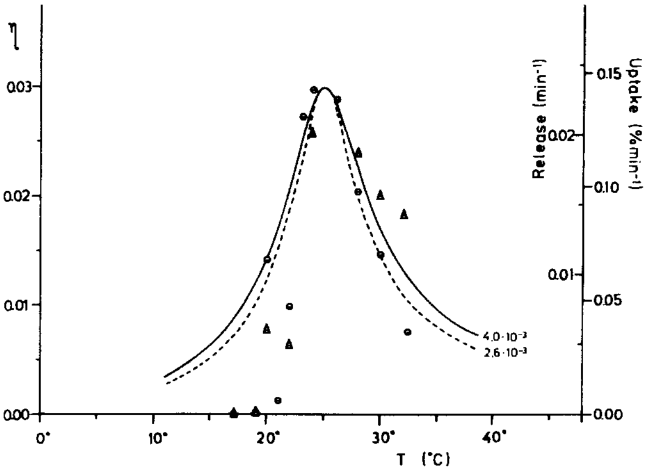

FIGURE 9: Comparison of the calculated fraction of interfacial lipid η, with the measured temperature profile of the permeability. Solid line calculated values of η for $\sigma = 4 \times 10^{-3}$; broken line: calculated values for $\sigma = 2.6 \times 10^{-3}$ (in the latter case the vertical scale must be multiplied by a factor of 0.82). (○) initial rate of uptake, (△) first-order rate constant for release.

structural phase transition (Figure 5c), respectively, is obvious. The center of the transition, T_t, is defined by the point at which $\theta = 0.5$ (cf. Figure 8b) and corresponds to $s = 1$ in eq 16. For small deviations about the center of the transition, the s dependence of Figure 8 can be transformed into a temperature-dependent scale by a Taylor Series expansion about T_t ($s = 1$):

$$s = 1 + \frac{\Delta H_t}{RT_t^2}(T - T_t) \qquad (18)$$

where ΔH_t is the molar enthalpy of transition, demonstrating that the s scale is linear in temperature for small deviations from T_t.

From Figure 8b it is clear that the width of the transition depends on σ; in fact, substitution in eq 16 shows that θ goes from 0.28 to 0.72 as s goes from $1 - \sigma^{1/2}$ to $1 + \sigma^{1/2}$, i.e., approximately 75% of the transition takes place within the range $\pm\sigma^{1/2}$. Substitution in eq 18 shows that the width of the transition over this range is given by:

$$\Delta T_t = 2\sigma^{1/2}\frac{RT_t^2}{|\Delta H_t|} \qquad (19)$$

Clearly, σ is an index of the cooperativity of the transition, the smaller σ, the greater is the degree of cooperativity, i.e., the narrower the transition. In addition, it may be shown that σ is directly related to the effective size of the cooperative unit undergoing the transition (Träuble, 1971; Tsong et al., 1970; Marsh et al., 1976a), and also to the mean size of the areas of ordered and fluid lipid shown in Figure 7 (Marsh et al., 1976a).

The value of σ can be determined from data such as that in Figure 5c, using the slope of the degree of transition curve in the region of the center of the transition. Differentiation of eq 16, using eq 18, yields:

$$\left.\frac{\mathrm{d}\theta}{\mathrm{d}T}\right|_{T_t} = \frac{1}{4\sigma^{1/2}}\cdot\frac{\Delta H_t}{RT_t^2} \qquad (20)$$

indicating that θ will have a linear dependence on $1/T$ in the region close to T_t, with a slope $-\Delta H_t/4R\sigma^{1/2}$, from which σ can be calculated.

Comparison with Experiment. Fitting the temperature dependence of θ deduced from the spin-label data of Figure 5c, with a plot of θ against $1/T$ as indicated by eq 20, yields a value of $\sigma = 4 \times 10^{-3}$, from the data for both spin-label II and III. A value of $\Delta H_t = 6.64$ kcal/mol is used for the calorimetric enthalpy of transition in these calculations (Chapman et al., 1967, Hinz and Sturtevant, 1972).

The values for the fraction of interfacial lipid, η, calculated using the above value of $\sigma = 4 \times 10^{-3}$ are compared with the temperature profile of the Tempo-choline permeability in Figure 9. The fit of the experimental data to the model is reasonably good, the actual experimental points varying somewhat more steeply with temperature below T_t. In this connection it should be emphasized that the value for σ is determined by a completely independent set of experiments. To indicate the degree of precision of the fit, values of η calculated for $\sigma = 2.6 \times 10^{-3}$ are also included. This value of σ represents the maximum upper limit on the slope of the degree of transition curves.

The correlation between the permeability and the fraction of interfacial lipid demonstrates that it is these latter regions that are responsible for the permeability and it is possible to use the values of η to calculate the intrinsic permeability of the interfacial regions (rather than the average permeability for the whole vesicle such as was calculated previously). At the center of the transition the intrinsic permeability of the interfacial regions is approximately 34 times greater than the vesicle permeability calculated above. Thus, the results from the uptake experiments (fast phase) are $P(T_t) = 1.0 \times 10^{-8}$ cm/s and $D(T_t) = 3.2 \times 10^{-15}$ cm²/s, and for the release experiment (slow phase) are: $P(T_t) = 2.3 \times 10^{-9}$ cm/s and $D(T_t) = 7.1 \times 10^{-16}$ cm²/s.

Discussion

The use of single-bilayer lipid vesicles of known dimensions has made it possible to measure the permeability coefficient to Tempo-choline. The values obtained for the vesicle permeability coefficient at the phase transition ($\sim 10^{-10}$ cm/s) are two orders of magnitude larger than those typically obtained for the permeability of black lipid membranes to the considerably smaller inorganic cations, Na^+ and K^+ ($\sim 10^{-12}$ cm/s, see, e.g., Jain, 1972). In this latter case, the lipids of the black lipid membrane are in the fluid phase.

The enhanced permeability is an intrinsic property of the vesicle and does not arise from vesicle fusion, since it was found, both by freeze-etch electron microscopy and by using the Taupin and McConnell (1972) protected volume assay, that negligible fusion took place with the incubation temperatures and times used here (Marsh, Watts, and Knowles, to be published). This latter result is in agreement with the findings of Kantor and Prestegard (1975), who concluded that their earlier report (Prestegard and Fellmeth, 1974) of rapid fusion of DMPC vesicles at the phase transition was due to an unidentified lipid contaminant.

The temperature variation results of Figure 5 demonstrate that the origin of the high permeability at the phase transition lies in the interfacial lipid at the boundaries of the regions of fluid and ordered phase that are assumed to coexist during the transition (see Figure 7). Since the Tempo-choline permeability is much smaller both above and below the transition, the enhanced permeability can be attributed neither to the fluid nor to the ordered phase lipid but arises from the areas of mismatch in molecular packing which occur at the interfacial regions between these two phases. Calculations of the fraction of interfacial lipid molecules, η, at various stages throughout the transition confirm this interpretation (Figure 9). The fit of the calculated values of η to the temperature profile is not exact, but the more rapid fall-off in permeability below the transition can be accounted for if it is assumed that the intrinsic permeability of regions at the interface of a small area of fluid lipid surrounded entirely by ordered lipid is not so high as in the reverse situation. The calculation that the intrinsic permeability of the interfacial regions at the center of the transition is over 30 times greater than the overall vesicle permeability indicates further how dramatically different the properties of the interfacial regions are from those of the ordered and fluid phases.

Similar results on the ion permeability of PC bilayers have also been obtained by other workers, in agreement with the above interpretation. Measurements of the K^+ permeability of multilayered liposomes of DMPC + 2% phosphatidic acid by Blok et al. (1975) have demonstrated a sharp peak in the permeability at the phase transition, with very low permeability above and below the transition. Measurements on dipalmitoylphosphatidylcholine bilayers (Blok et al., 1975; Papahadjopoulos et al., 1973; Nichols and Miller, 1974; Wu and McConnell, 1973) have shown that the ion permeability exhibits a small, relatively sharp peak at the phase transition, although this peak is considerably smaller than for DMPC liposomes (Blok et al. 1975). In addition, Blok et al. (1975) have demonstrated a K^+ permeability maximum in the lateral phase separation region of DMPC–dipalmitoylphosphatidylcholine mixed bilayers.

It is possible, to extend the above analysis to mixtures of lipids under conditions of lateral phase separation by using eq 19 to estimate σ from the width of the lateral phase separation region. From consideration of Figure 8a, it is to be expected that the proportion of the interfacial lipid will be much greater in systems exhibiting lateral phase separation, since the cooperativity of the transition is significantly lower. For equimolar mixtures of phosphatidylcholines differing in chain length by only two carbon atoms (which show cocrystallization: Shimshick and McConnell, 1973; Chapman et al., 1974; de Kruyff et al., 1974) a value of $\eta(s = 1) \simeq 0.05$ is obtained for the fraction of interfacial lipid at the center of the transition. This is to be compared with the value of $\eta(s = 1) \simeq 0.007$ for the single phospholipid (Hinz and Sturtevant, 1972). For equimolar mixtures of phosphatidylcholines with phosphatidylethanolamines of equal chain length (Shimshick and McConnell, 1973; Chapman et al., 1974; Blume and Ackermann, 1974) the value is even larger: $\eta(s = 1) \simeq 0.10$. These relatively high values of η indicate that phase boundary lipid will probably be important in determining the properties of lipid mixtures under conditions of lateral phase separation, although the mismatch in molecular packing might not, however, be so acute in the mixed systems as that found with the single lipids.

Concerning the biological significance of the interfacial lipid, it is possible that the sharp increase in permeability at the phase transition could be a mechanism for the release of chemical transmitters, (e.g., acetylcholine or adrenaline which are of comparable size and charge properties to Tempo-choline), or possibly even enzymes, from secretory granules. In this connection it is relevant to note that a structural transition, attributed either to a phase transition or some form of phase separation, has recently been demonstrated to occur in the lipid phase of the membrane of the chromaffin granule which is responsible for the storage and release of adrenaline from the adrenal medulla (Bashford et al., 1976; Marsh et al., 1976b). A further implication could be in the function of proteins embedded in the lipid phase of the membrane. The peculiar packing properties of the interfacial lipid could facilitate conformational changes within proteins; the reported twofold *increase* in sugar transport rate of *E. coli* fatty acid auxotrophs resulting from a 1 °C *decrease* in temperature from the fluid to the phase-separation region (Linden et al., 1973) could be the result of such a mechanism. The elegant experiments of Op den Kamp et al. (1975) on the temperature dependence of the rate of hydrolysis of phospholipids by porcine pancreatic phospholipase A_2, which were interpreted as showing that the enzyme could only penetrate the lipid bilayer in the interfacial lipid regions, also indicate the possibility of such a mechanism. Taken together with the calculations of the fraction of interfacial lipid in the present paper, these latter experiments provide a convincing demonstration of the possible importance of phase boundary lipid in the functioning of biological membranes.

Finally it should be pointed out that there are now several reports of lipid phase transitions taking place at physiological temperatures in biological membranes, e.g., the chromaffin granule membrane system quoted above (Bashford et al. 1976; Marsh et al., 1976b), the cytochrome P450-reductase system in liver microsomes (Stier and Sackmann, 1973) and the lung surfactant system (Träuble et al., 1974). However, it is likely that the results of this paper will be of considerably greater generality, since there are many other interfaces within biological membranes at which a mismatch in molecular packing could occur. Packing defects at the interfaces between different domains of ordered phase lipid have already been suggested to occur (Lawaczeck et al., 1975) and of most importance are the possible functional implications of molecular mismatch at lipid–protein interfaces.

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