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DYE PERMEABILITY AT PHASE TRANSITIONS IN SINGLE AND BINARY COMPONENT PHOSPHOLIPID BILAYERS

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By encapsulating a pH-sensitive dye, phenol red, in multilamellar liposomes of DMPC, DPPC and DMPC/DPPC mixtures, the permeability of these phospholipid bilayers to dye as a function of temperature has been studied. For both DMPC and DPPC liposomes, dye release begins well below the main gel-to-liquid-crystalline phase transition (24°C and 42°C, respectively) at temperatures corresponding to the onset of the pretransition (about 14°C and 36°C, respectively) with DPPC liposomes exhibiting a permeability anomaly at the main phase transition (42°C). The perturbation occurring in the bilayer structure that allows the release of encapsulated phenol red (approx. 5 Å diameter) is not sufficient to permit the release of encapsulated haemoglobin (approx. 20 Å diameter, negatively charged). In liposomes composed of a range of DMPC/DPPC mixtures, dye release commences at the onset of the pretransition range (determined by optical absorbance measurements) and increases with increasing temperature until the first appearance of liquid crystalline phase after which no further dye release occurs. Interestingly, the dye retaining properties of DMPC and DPPC liposomes well below their respective pretransition temperature regions are very different: DMPC liposomes release much encapsulated dye at incubation temperatures of 5°C whilst DPPC liposomes do not.

Introduction

Enhanced permeability of model membranes at their phase transition temperature has been studied extensively for pure lipids [1]. In general bilayer permeability increases with decreasing hydrocarbon chain length and with the degree of unsaturation of the chains. In particular permeability is enhanced in the region of the phase transition and the onset of the increase begins several degrees below the main phase transition temperature t_M [2]. In a study of glucose diffusion through a membrane [3], constituted from a mixture of mainly DMPC/DPPC, glucose release extended over a range of about 12°C which is much broader than the accepted gel plus liquid-crystalline region indicated by the phase diagram [4]. In addition, and perhaps not surprisingly, a pretransition at a lower temperature t_L is observed in lipid mixtures [5] as for the pure lipids. However, the nature of the pretransition, even for pure lipids, is not well understood although it is commonly accepted that it is associated with a change in long range order [19,22], which subsequently disappears completely at the main transition.

There is some variability in the results obtained with different model systems. The phase transition behaviour of multilamellar vesicles and small unilamellar vesicles are very different in that, for

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example, DSC measurements of DPPC multilamellar liposomes [6] show a sharp, highly cooperative main transition with a somewhat broader pretransition whilst NMR measurements on small (sonicated) unilamellar vesicles of DPPC show a single, broad transition, shifted to lower temperatures [7]. Ideally, for permeability studies as a function of temperature, large unilamellar vesicles should be used since these are the most realistic models for cell membranes. However, the fabrication of large unilamellar vesicles is not trivial and so multilamellar liposomes were used, being easier to prepare and having similar phase transition properties to large unilamellar vesicles. Using multilamellar structures with aqueous phase trapped between lamellae precludes the calculation of dye leakage rates, since many bilayers separating many different aqueous compartments are involved, and only a measure of the gross dye leakage is obtained. Although small unilamellar vesicles are attractive, in that they have a single bilayer enclosing an aqueous space, they were not used here since they are most probably in a state of metastable equilibrium [8] and experiments have shown that the rate of fusion of small unilamellar vesicles (both for DMPC and DPPC) is maximal in the phase transition region [9].

The present work was designed to study dye permeability through multilamellar vesicles of pure DMPC and DPPC, and mixtures of the two, in the phase transition region. In particular it is shown that the onset of permeability is linked to the pretransition in these bilayers.

Materials and Methods

Sample preparation

DMPC and DPPC (in powdered form, approx. 98% pure) were obtained from Sigma Chem. Co. Ltd. and used without further purification. Multilamellar liposomes were prepared from about 10 mg of pure lipids and lipid mixtures using standard lipid cast techniques in a 50 ml round bottom flask. Phenol red dye was incorporated in the vesicles by adding a 5 mg/ml solution of the sodium salt of phenol red (Sigma Chemical Co. Ltd.) to the flask before removal of the cast. To remove the non-encapsulated dye the resultant dark red, very cloudy, preparation of multilamellar liposomes was passed through a chilled Sephadex G-25M column whence liposomes eluted first (with cold buffer) as a cloudy yellowy-orange fraction. In order to have the temperature well below the pretransition temperature ranges of DMPC and DPPC, during dye removal, the column (Pharmacia, pre-packed, bed height 5 cm and volume 9.1 ml) was previously chilled by passing water at 5°C through a surrounding water jacket, and equilibrating the column with buffer at 5°C for an hour before use. The liposome preparation was also chilled prior to placing on the column and then aliquots of 500 μl applied to the top of the column through a head of cold buffer. On being eluted, the 'multilamellar vesicle encapsulated-dye' fraction was collected in a small amount of cold buffer (5°C), further diluted to 0.2 mg lipid/ml buffer, and stored on ice for the duration of the experiment.

Haemoglobin encapsulation

Haemoglobin (Type 1 from bovine blood, Sigma) was reconstituted in Drabkins solution (contains sodium bicarbonate, potassium ferricyanide, potassium cyanide) to a concentration of 12 mg/ml and centrifuged at $750 \times g$ for 10 min to remove any undissolved haemoglobin. The resultant supernatant (dull red, clear) had an approximate concentration of 10 mg/ml, the haemoglobin existing mostly as cyanmethaemoglobin (negatively charged). 1 ml of this haemoglobin solution was added to a lipid cast (20 mg DPPC containing 4% by weight of negatively charged phosphatidic acid) and multilamellar liposomes formed as previously described. The haemoglobin external to the multilamellar vesicles was removed by spinning the preparation at 2000 $\times g$ and 5°C for 20 min on a refrigerated Sorvall RC 3 B centrifuge and discarding the clear supernatant above the resultant liposome sediment. 20 ml of 10 mM Tris buffer (pH 7.0) was added, and the liposomes resuspended with vigorous shaking. This 'centrifugation-washing' procedure was repeated three times, the final lipid concentration being made up to 0.5 mg/ml.

Experimental procedure

The experimental apparatus used for the dye leakage experiments was a double beam mono-

chromatic ($\lambda = 550$ nm) photometer. The reference cuvette temperature was kept at 5°C and was continuously monitored with a Ni-Cr/Ni-Al thermocouple placed directly in the liposome preparation.

Determination of dye leakage

The sample cuvette contents were first rapidly heated from 10°C to the desired temperature and then cooled back rapidly to 10°C 5 min after the onset of the temperature rise. Heating and cooling rates of 30 to 40 (K/min) were obtained; several cold-hot-cold cycles are shown in Fig. 1, the temperature in the continuously stirred preparation also being measured by a Ni-Cr/Ni-Al thermocouple.

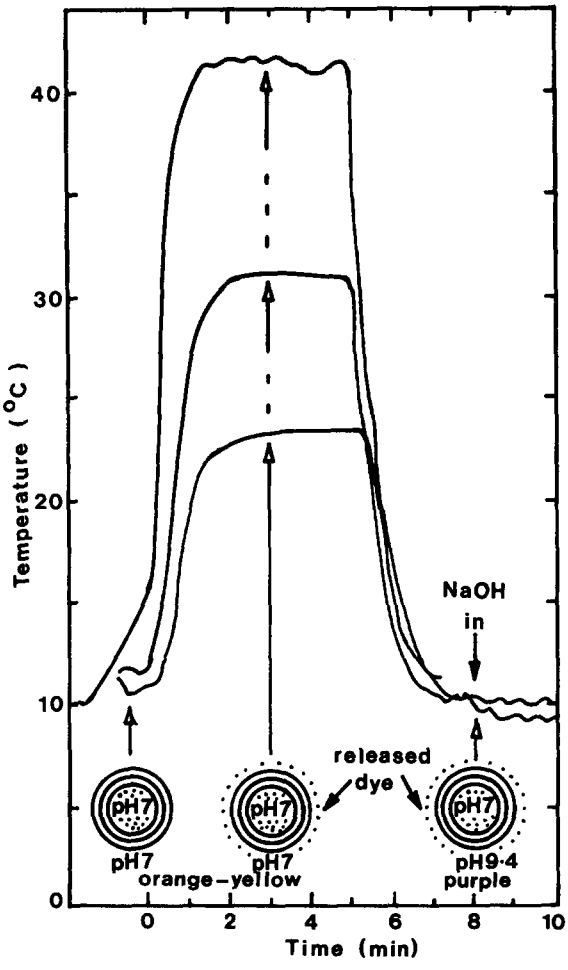

Fig. 1. Typical cold-hot-cold cycles for liposome preparations with the corresponding pH regime shown below.

After allowing the heated-cooled preparation to equilibrate at 10°C, 100 μl of 0.05 M NaOH was injected (a total of 8 min after the onset of the temperature rise) into the sample cuvette.

This shifts the pH external to the liposomes from 7 to 9.4 and any dye released from the liposomes undergoes a distinct colour change from orange-yellow to red-purple. The low and high pH forms of phenol red have absorption maxima at 420 and 550 nm, respectively, with the pH dependence shown in Fig. 2.

It was necessary to adopt the above procedure for measuring dye leakage from multilamellar vesicles as a function of temperature because multilamellar liposome preparations undergo large turbidity changes in the phase transition region and because the buffer pH decreases with increasing temperature. The NaOH is added after the temperature has been returned to 10°C in order to ensure that there is no pH gradient across the membrane during the temperature excursion. This ensures that changes observed are associated with dye leakage and not with permeability to H^+ or OH^-.

Measurement of cyanmethaemoglobin release

Cold-hot-cold cycles were performed on 3-ml aliquots of multilamellar vesicles encapsulating haemoglobin as described in the previous section. The multilamellar liposomes in these heated-cooled

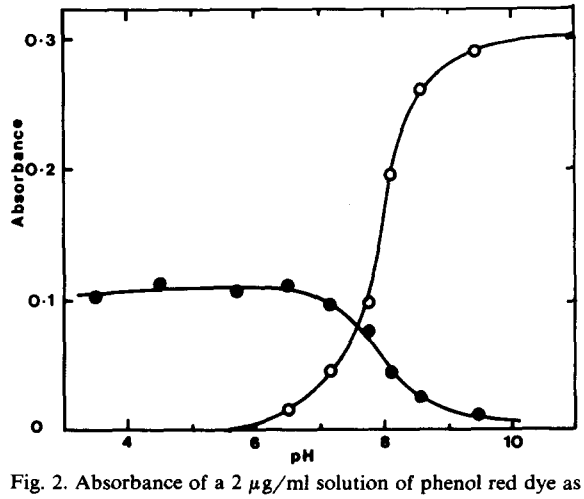

Fig. 2. Absorbance of a 2 μg/ml solution of phenol red dye as a function of pH: ●, for light of wavelength 420 nm; ○, for light of wavelength 550 nm.

preparations were spun down (2000 × *g* for 20 min at 5°C) and the amount of haemoglobin released estimated by measuring the supernatant absorbance at 276 nm.

For each heated-cooled preparation, the amount of haemoglobin remaining encapsulated within the liposomes was measured by resuspending the sediment with vigorous shaking, lysing the liposomes by warming in the presence of a small amount of Triton X-100 and measuring the absorbance at 276 nm.

Results

Dye retaining properties of DMPC and DPPC

3-ml aliquots of DMPC and DPPC multilamellar vesicles encapsulating dye at 5°C were transferred into cuvettes and a 100 μl of 0.05 M NaOH added to each, thus raising the pH external to the liposomes to pH 9.4. The liposomes were spun down by centrifuging the cuvettes at 2000 × *g* for 20 min at 5°C and the absorbance (A) of the supernatant for each cuvette measured at 550 nm. The values obtained were 0.32 (orange colour) and 0.94 (distinctly purple) for DPPC and DMPC supernatants, respectively, indicating that, although kept at 5°C, the DMPC liposomes had released much of their encapsulated dye over a period of about one hour. Also the DMPC and DPPC liposome sediments were purple and orange in colour, respectively, showing that for DMPC multilamellar vesicles the pH gradient across the liposomal bilayers had equilibrated. The liposome sediment in each cuvette was then resuspended by shaking and the liposomes lysed by warming in the presence of Triton X-100. On lysing the DPPC preparation underwent a strong colour change from orange to purple whereas the DMPC sample showed no visible colour change. The absorbance values of the lysed multilamellar vesicle preparations (A') were 1.1 and 1.0 for DPPC and DMPC preparations, respectively. The quantity $A' - A$ is a measure of the dye encapsulated (or retained) within the liposomes and is approx. 0.80 and 0.04 for DPPC and DMPC multilamellar vesicles, respectively.

This result shows that at 5°C, and well below their pretransition temperature, DMPC liposomes fail to maintain their integrity as 'sealed' capsules and release most of their dye. DPPC liposomes, however, are not leaky at low temperatures.

Captured volume determination

The captured volume for DPPC multilamellar vesicles may be calculated using the difference ($A' - A$) which, for a sample of length x, is given by $\varepsilon c' x$ where ε is the dye absorption coefficient at pH 9.4 and c' the increase in the supernatant dye concentration due to encapsulated dye being released from multilamellar vesicles on lysis. For phenol red ε, at pH 9.4 and 550 nm, was found to be $3.8 \cdot 10^6\ M^{-1} \cdot cm^{-1}$ (approx. 108 $ml \cdot mg^{-1} \cdot cm^{-1}$) from the gradient of a linear plot of absorbance versus dye concentration at pH 9.4 and 550 nm.

Hence c' was calculated to be 6.8 μg/ml of liposome preparation. Since the dye concentration within the liposomes is 5 mg/ml and the liposome preparation concentration 0.2 mg/ml, then the captured volume is (6.0 ± 1.0) μl/mg of lipid. This value compares well with the value of 4.1 μl/mg given by Szoka and Papahadjopoulos for multilamellar vesicles composed of egg PG, PC and cholesterol in the ratio 1 : 4 : 5.

Dye release from DPPC multilamellar vesicles

Fig. 3(a) shows a graph of change in absorbance (at 550 nm on the addition of NaOH at 10°C after various heating-cooling cycles) versus incubation temperature for DPPC multilamellar liposomes encapsulating phenol red.

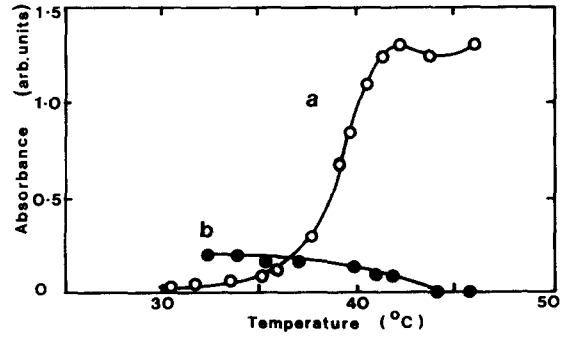

Fig. 3. Change in 550 nm absorbance on addition of NaOH versus incubation temperature for DPPC multilamellar liposomes encapsulating phenol red dye. (a) Cold-hot-cold cycles with hot incubation for 5 min and NaOH added at the end of each cycle (10°C). (b) Hot-cold-hot cycles with cold incubation for 5 min and NaOH added at the end of each cycle (50°C).

At temperatures below 35°C, dye release does not occur. However, the amount of encapsulated dye released increases dramatically between 35°C and 42°C, exhibiting an anomaly around 42°C. Several observations of dye release from multilamellar DPPC liposomes as a function of temperature gave similar results.

In order to verify the existence of a permeability anomaly at t_M, dye release experiments on cooling through the phase transition were performed (i.e. hot-cold-hot cycles).

DPPC multilamellar liposomes (approx. 0.4 mg/ml) encapsulating dye were kept well above t_M during the external dye separation procedure (by using a heated Sephadex column) and the resultant preparation kept at 50°C in a hot water bath for the duration of the experiment.

Samples were cooled to various temperatures from 50°C, and then heated again to 50°C after the incubation period and NaOH added. As a control, immediately after each cooling-heating cycle, the absorbance change on adding NaOH to a sample kept at 50°C was recorded. The control shows a large change in colour right at the outset of the experiment which increased gradually with time as the experiment progressed, indicating that above t_M DPPC multilamellar liposomes are leaky. A contribution to the colour change obtained on adding NaOH above t_M may be due to the partial equilibration of the pH gradient created across the liposome bilayer(s).

Fig. 3(b) shows the difference in absorbance values versus incubation temperature obtained for the hot-cold-hot cycled samples and the control on the addition of NaOH. This shows a small but definite increased leakiness in DPPC multilamellar liposomes on cooling through the phase transition region. The smallness of this increase is probably due to the small amounts of dye remaining encapsulated in DPPC liposomes at 50°C so that cooling to below t_M results in little contribution, through increased leakage at t_M, to the amount of dye already present outside the liposomes.

Determination of the 'hole' size

Given that dye encapsulated within multilamellar vesicels is released in the phase transition region it is of interest to determine the approximate size of the 'holes' occurring in the lipid bilayers, on the assumption that multilamellar vesicles are stable in the t_M region and do not rupture causing non-specific release of contents. The experiments were therefore repeated with the encapsulation of larger molecules (haemoglobin).

The absorbance (at 276 nm) of liposomes over the temperature range 23°C to 53°C remained constant at 0.05 ± 0.02 while, in contrast, the protein released on lysing the resuspended liposomes after each cold-hot-cold cycle yielded absorbance values in the range 0.8 ± 0.2. There was no significant trend with temperature. These data indicate that little encapsulated haemoglobin is released at any temperature, although the total amount of haemoglobin encapsulated is large, with no evidence of an increase in protein release in the phase transition region. Hence any perturbations that may be occurring in the bilayer structure are insufficient to permit the release of this large, negatively charged, protein molecule.

Dye release from DMPC/DPPC multilamellar vesicles

Fig. 4 shows a graph of change in absorbance (on addition of NaOH at 10°C) after various cold-hot-cold cycles for some mixtures of DMPC/DPPC and DPPC multilamellar liposomes encapsulating dye. The relatively flat portion exhibited by the mixtures after the maximum permeability region may be ascribed to an equilibration of the dye gradient across the liposomal bilayer(s) since, for example, DPPC multilamellar liposomes are leaky to dye above t_M. DMPC multilamellar liposomes (not shown in Fig. 4), because of their poor dye-retaining properties in the gel

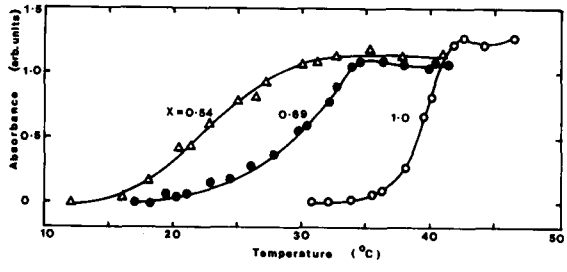

Fig. 4. Change in 550 nm absorbance versus incubation temperature for cold-hot-cold cycles of DMPC/DPPC multilamellar vesicles encapsulating phenol red dye. NaOH is added at the end of each cycle (10°C). X, molar fraction of DPPC.

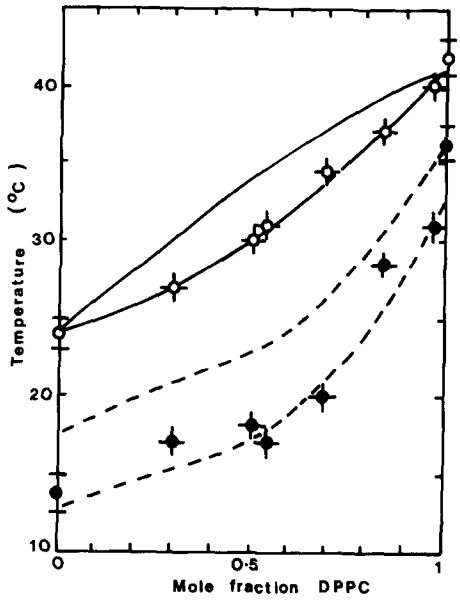

Fig. 5. Phenol red dye permeability in relation to the DMPC-DPPC phase diagram. ●, onset of dye permeability; ○, completion of region of increasing permeability. The solid curves show the solidus and liquidus lines for DMPC/DPPC mixtures as derived in separate experiments using a light scattering technique. The broken curves mark the beginning and end of the pre-transition region determined in the same way.

phase, exhibit only a small amount of dye release (approx. 0.2 absorbance units) in the range 14°C to 24°C.

We can obtain a measure of the range of the enhanced permeability region by taking the temperatures at which the absorbance in Fig. 4 begins to depart from the approximate linearity apparent below and above t_M. The temperatures that define the onset and completion of the maximum permeability region are shown plotted versus mole fractions of DPPC in each mixture in Fig. 5 for a range of mixtures.

Discussion

Fig. 3(a) shows that for multilamellar DPPC liposomes the permeability to phenol red molecules begins to rise in the pretransition region (35°C) and exhibits a permeability anomaly around t_M. The measurements are of total dye leakage during a complete heating-cooling cycle, that is the summed dye leakage on rapid heating (heating rate 30–40 $K \cdot min^{-1}$), subsequent incubation at a particular temperature, and rapid cooling back to 10°C. It should be stressed therefore that the approximately flat portion in Fig. 3(a) above 42°C indicates that no further dye leakage takes place on increasing the temperature in this region. In view of the experimental protocol it is possible that this effect could be associated with either a permeability maximum at t_M or the fact that internal and external dye concentrations had equilibrated around t_M and no further leakage was possible. The results of experiments in which t_M was approached from within the liquid-crystalline phase, however, indicate that the permeability does indeed fall for temperatures above t_M. These results are in general accord with previous measurements on the permeability of single component vesicles to different permeants. It should be noted in connection with the present experiments that at pH 7.0, the phenol red molecules are 75% in the neutral state and 25% in the negatively charged state.

The observation that encapsulated haemoglobin is not released on passing through t_M indicates that the perturbations in the bilayer are large enough to allow phenol red (about 5 Å) through, but not haemoglobin (about 20 Å). It would clearly be of interest to further investigate this 'pore' size by studying the release of encapsulated proteins in a size range intermediate between these two values.

A permeability peak at 42°C for electrolytes and nonelectrolytes in DPPC liposomes, both sonicated and multilamellar, has been demonstrated by many workers [1,10,11]. The results for small unilamellar vesicles (sonicated) should be regarded with caution since small unilamellar vesicles are not stable structures and tend to fuse, the fusion rate being maximal at the phase transition and large above t_M. For example, Haest et al. [12] showed that maximal K^+ release was obtained around 23°C, in DMPC (plus about 4% phosphatidic acid) liposomes entrapping potassium. Papahadjopoulous et al. [10] found the self-diffusion rates of $^{22}Na^+$ from DPPC multilamellar liposomes to be increased from 35°C and reach a maximum at 42°C. Similar results were obtained by Blok et al. [1] for K^+ efflux (and also for a variety of other ions) from DMPC and DPPC

liposomes, the permeability at the phase transition temperature being determined by the size of the permeant ion and by the length of the lipid hydrocarbon chains.

It has been suggested [10,11] that this permeability peak is associated with the formation of 'domains' with enhanced diffusion taking place at domain boundaries. Although attractive for lipid mixtures, such a theory has not gained widespread acceptance because of the difficulty of applying it to single component systems. Doniach [13] and Nagle and Scott [14] on the other hand suggest that the peak is due to enhanced fluctuations in lateral density. Such fluctuations are associated with high lateral compressibility due to the fact that the membrane is near to a critical point. Thus increased permeability is caused by the opening of short-lived cavities in the headgroup region.

Although the onset of increased permeability in DPPC coincides with the pretransition this point has not been accommodated in any theoretical analysis. To investigate this coincidence more carefully we have carried out a complete study of the phase diagrams for a number of two component lipid vesicles systems [15]. The samples were prepared in an identical manner to that described above and a simple light scattering technique used to determine phase boundaries.

For the DPPC/DMPC system we have been able to plot the well known solidus and liquidus curves [4], but in addition we have observed the pretransition for all lipid compositions. In Fig. 5 we plot as solid lines our experimentally determined solidus and liquidus curves as well as the beginning and end of the pretransition region for each composition. It is evident that, for both single and two-component multilamellar liposomes, dye release commences at the onset of the pretransition and increases with increasing incubation temperature until the first appearance of liquid-crystalline phase (the solidus curve) after which temperature no further increase in dye release occurs. The implication of this result is that the permeability peak is unlikely to be associated with the boundary lipid in the gel-liquid crystal two-phase region. Rather it is suggested that the changes in bilayer structure at the pretransition accelerate permeability increases associated with density fluctuations.

Studies by Inoue [3] on the diffusion of glucose from liposomes prepared with DMPC/DPPC mixtures (with about 0.10 mole fraction of dicetyl phosphate present) show that for liposomes containing equimolar amounts of DMPC and DPPC, glucose release commences around 25°C, with the highest permeability being reached at about 37°C. For liposomes containing DPPC/DMPC/dicetyl phosphate in the molar ratios (0.75 : 0.25 : 0.1), the corresponding temperatures are 28°C and 37°C, respectively. From the DMPC/DPPC phase diagram (and assuming that the phase diagram for DMPC-DPPC-dicetyl phosphate is similar) it appears that glucose release commences in the pretransition region and several degrees below the formation of the first liquid-crystalline phase in general agreement with the dye release results.

The nature of the pretransition in bilayers is still a matter of some speculation. Below the pretransition temperature t_L the molecules are in a predominantly all-*trans* state and pack closely in a regular lattice, with chains all aligned but in some cases tilted relative to the bilayer normal. In DPPC this tilt is 30° [16]. For temperatures just above t_L Janiak et al. [17] report that ripples appear which persist up to t_M. The appearance of the ripples at t_L coincides with the disappearance of the tilt angle [18]. An alternative picture is provided by Inoko et al. [19] who show evidence that the lipid thickness does not change at t_L and that the ripple phase is only seen in the absence of excess water. Hence they conclude that the chain tilt angle does not change at t_L. Furuya and Mitsui [20] interpret their X-ray measurements in terms of the appearance above t_L of a set of domains whose tilt angles have a common value with respect to the bilayer normal, but vary azimuthally between each other. Recently, however, Stamatoff et al. [21] have confirmed Janiak's observation of an untilted ripple phase between t_L and t_M. Despite this conflicting evidence it appears that the pretransition is associated with the onset of rotation about the molecular long-axis. Scott's model [22] in which the hydrocarbon chains are treated as elongated projections on to the membrane plane is particularly illuminating. The packing mismatch of rotated elongated molecules suggested by this model provides a mechanism by which membrane permeability might increase from t_L upwards. The 'tilted

domain model' is not necessarily inconsistent with this picture and provides an additional source of molecular mismatch at domain boundaries. The nature of the ripple phase has been studied in detail by Stamatoff et al. [21] who find a ripple wavelength of about 16 molecules and a ripple amplitude of approximately one hydrocarbon chain length. On this evidence it would seem that the suggested ripples are sufficiently gentle not to lead to 'holes' in the membrane structure. It appears therefore that the most likely mechanism of increased permeability is long axis rotation on either an individual molecule or a 'domain' level as discussed above.

Conclusions

The permeability of DPPC and DMPC/DPPC lipid bilayers to phenol red dye molecules increases rapidly in the region between the pre-transition and main transition for vesicles of these materials. The increased permeability is associated with the appearance of pores in the headgroup region between 5 Å and 20 Å in diameter. It is suggested that these pores are associated with the onset of long axis rotation at the pretransition, in conjunction with lateral density fluctuations in the bilayer.

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