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THE FORMATION AND ANNEALING OF STRUCTURAL DEFECTS IN LIPID BILAYER VESICLES

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SUMMARY

It is shown that sonication of phospholipid-water dispersions below the crystalline → liquid crystalline phase transition temperature (T_c) produces bilayer vesicles with structural defects within the bilayer membrane, which permit rapid permeation of ions and catalyze vesicle-vesicle fusion. These structural defects are annihilated simply by annealing the vesicle suspension above T_c. The rate of annealing was found to be slow, of the order of an hour for $T = 3$ °C above T_c, but annealing is complete within 10 min for $T = 10$ °C above T_c. It is proposed that these structural defects are fault-dislocations in the bilayer structure, which arise from a population defect in the distribution of the lipid molecules between the outer and inner monolayers, when small bilayer fragments reassemble to form the small bilayer vesicles during the sonication procedure. Such a population defect can only be remedied by lipid transport via the inside ⇌ outside flip-flop mechanism, which would account for the slow kinetics of annealing observed even at 3 °C above the phase transition.

INTRODUCTION

Hydrated phospholipids in their multilamellar state and sonicated single-walled vesicles offer two different, albeit interrelated, model membrane systems, each with some unique advantages in membrane studies. In terms of bilayer structure, these two model systems differ primarily in their surface curvatures [1]. Unsonicated multilayers, by virtue of their larger size, bear with respect to their surface curvature the greater resemblance to biological cell membranes and artificial black lipid films. However, their onion-like structure render them not suitable for studies of transport

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phenomena. On the other hand, since vesicle curvature can have a profound influence on the molecular packing of the phospholipid molecules in a bilayer, small sonicated bilayer vesicles 250–300 Å in diameter may or may not be relevant with respect to biological membranes. In these small structures, the available volume for each phospholipid molecule deviates drastically from ideal hexagonal close packing due to the "wedge" effect [2]. In fact, the inner and outer bilayer surfaces have quite different surface curvatures, both in magnitude and sign. Small sonicated bilayer vesicles are, therefore, intrinsically asymmetric, and this membrane asymmetry between the two halves of the bilayer is no doubt responsible for the structural differences which have been observed between the two monolayers and the asymmetric lipid distribution which have been reported for bilayer vesicles containing two lipid components [3, 4]. In view of the large surface curvature, small bilayer vesicles are also expected to be intrinsically less stable thermodynamically than their unsonicated counterparts. In fact, there is now considerable experimental evidence indicating that small sonicated bilayer vesicles do tend to undergo vesicle-vesicle fusion [5–8] in order to dissipate this excess surface free energy.

Despite the above disadvantages, single-walled bilayer vesicles offer the distinct advantage of having a well-defined inside and outside [9] and are therefore particularly suitable for membrane transport and permeability studies [10]. For this reason, we have devoted some effort towards developing a procedure for obtaining single-walled bilayer vesicles which are of the order of approx. 1000 Å or larger in diameter and accordingly would be less perturbed by curvature effects. Larger bilayer vesicles can be prepared by mild sonication followed by size fractionation by column chromatography. However, this procedure is quite time-consuming and we have found that the results are not always reproducible. An alternate method could conceivably be developed based on the controlled fusion of small sonicated vesicles. Recently, we reported from this laboratory that small sonicated phospholipid bilayer vesicles which are prepared by intermittent sonication so that the local temperature of the solution remains well below the thermal phase transition (T_c) of the lipid under consideration contain structural defects and exhibit a pronounced tendency to fuse near T_c [11]. These vesicles, which are also leaky to ions, can be rendered stable towards fusion and ion permeation merely by annealing the vesicle suspension above the phase transition temperature. Thus, larger single-walled bilayer vesicles can in principle be prepared by the fusion of small sonicated bilayer vesicles which contain these defects at a temperature near but below T_c, followed by rapid annealing of the remaining defects at temperatures sufficiently well above the lipid phase transition temperature.

The purpose of the present work is to clarify the nature of the structural defects in small bilayer vesicles which have been produced by sonication below T_c as well as the details of the vesicle-vesicle fusion and annealing process associated with these structural defects, so that a method for the preparation of large single-walled bilayer vesicles can be developed and perfected based on these findings.

EXPERIMENTAL

L-α-Dimyristoyl phosphatidylcholine and L-α-distearoyl phosphatidylcholine were purchased from Calbiochem; cholesterol and sodium cholate were from Grand Island. L-α-dipalmitoyl phosphatidylcholine was obtained from Calbiochem and

Grand Island, and lipids from both sources, were shown to give the same results described in the text. These compounds did not show detectable impurities by thin-layer chromatography, and were mostly used without further purification. For some experiments, however, distearoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine were purified by column chromatography on silicic acid (Unisil, 200-323 mesh) to remove possible traces of impurities. Reasonable candidates for these impurities are supposed to be fatty acids and lysophosphatidylcholine. These purified phospholipids also showed a single spot in their thin-layer chromatography, but behaved slightly differently from the lipids without purification. We shall refer to these purified compounds as purified dipalmitoyl phosphatidylcholine or distearoyl phosphatidylcholine.

The lanthanide and other inorganic salts were products of Research Organic/Inorganic Chemical Corp., and F. T. Baker Chemical Co., respectively. Tris(tri(hydroxymethyl)aminomethane) was purchased from Sigma. All salts were lyophilized from deuterium oxide (Stohler Isotopic Chemicals 99.8 %) to reduce the residual water peak.

The preparation of the vesicle suspension was carried out as follows. An appropriate amount of lecithin was weighed into small test tubes and after addition of usually 1 or 2 ml of water (^2H_2O) or an aqueous salt solution, the solution was shaken by a Vortex mixer and heated above the phase transition temperature of the lipids until a homogenous milky suspension was obtained. The test tube was then placed into a Branson sonicator with Ti-microtip and cooled down by an ice-water bath to around 0 °C. Either a high power interval-sonication of 30 s sonication and 30 s waiting for a total duration of 30 min, or a low power continuous sonication for 15 min was used. In both cases the solution was extensively cooled and maintained below the thermal phase transition temperature of the lipid. As the local microtip temperature is expected to be actually different from the cooling bath, and may reach quite high values during the sonication, special care was taken in the case of dimyristoyl phosphatidylcholine to prevent the solution from reaching a temperature higher than approx. 23 °C, the T_c for this lipid. After sonication, the solution was centrifuged to remove any Ti particles and filtered through a cotton layer. The remaining vesicle dispersion was clear, typically bluish and opalescent for trivalent cation salt solutions. The turbidity increased for solutions containing divalent cations and drastically for those with monovalent cations or in pure water. These observations may be contrasted with the results obtained for sonication above T_c, where a clear, bluish and waterlike solution is always obtained.

From these stock vesicle dispersions, usually of 1–5 % (w/v) lipid concentration in typically 5 mM $La(NO_3)_3$, 0.2 ml were pipetted into 5 mm diameter nuclear magnetic resonance sample tubes and then incubated for fixed times at various temperatures. After this incubation period the samples were cooled down to room temperature (23 °C) or in the case of dimyristoyl phosphatidylcholine to 0 °C, and either 0.2 ml aqueous 5 mM $Eu(NO_3)_3$ or $La(NO_3)_3$ (in the control sample) were added. The samples were gently, but thoroughly, mixed, and kept at the temperature of the nuclear magnetic resonance probe (usually above T_c of the respective lipids) prior to the nuclear magnetic resonance measurements for reasons which will be discussed below. Unless otherwise stated, mixed lipids were weighed together, dissolved in chloroform to allow complete mixing, lyophilized, and then handled as described above.

Proton magnetic resonance spectra were obtained at various temperatures using a Varian HR 220 or XL-100 spectrometer operating in the FT mode. The spectral width was 2.5 kHz for temperatures $> T_c$ and 5 kHz for temperatures $\leq T_c$. Usually each set of experiments included 5 to 8 samples and the nuclear magnetic resonance data accumulation, repetition rate, and spectral plotting parameters were kept constant for each set.

Absorbance measurements at $\lambda = 600$ nm were performed on a Beckman DU spectrometer. For these measurements, the stock solutions were diluted to obtain a convenient absorbance reading (approx. 0.1–0.5 absorbance units). The absorbance was measured as a function of temperature by increasing or decreasing the temperature of the cuvettes approx. 0.5 °C/min and simultaneously monitoring the temperature with a Copper-Constantan thermocouple.

RESULTS AND DISCUSSION

Formation and characterization of leaky dipalmitoyl phosphatidylcholine vesicles

When dipalmitoyl phosphatidylcholine vesicles are prepared by sonication below T_c, we obtained rather surprising and partly irreproducible results. The proton magnetic resonance spectra of these vesicles in the presence of Eu^{3+} (added isotonically after sonication and below T_c) showed only one choline methyl signal, which was shifted upfield compared to a control sample which contained only La^{3+} in both the intra- and extravesicular media (compare spectra A and B in Fig. 1). * Since the Eu^{3+} was added to the dipalmitoyl phosphatidylcholine dispersion at room temperature in these experiments, a logical next step was to mix the Eu^{3+} shift reagent with the vesicle solution at higher temperatures ($T > T_c$). This resulted in two well separated choline methyl resonances for the inside and outside monolayers. Finally we introduced a preheating of the vesicle solution at various temperatures both below and above T_c prior to mixing with the Eu^{3+} solution at room temperature. Typical results obtained in these experiments are summarized in Fig. 1 for dipalmitoyl phosphatidylcholine (purified) vesicles. These spectra were recorded above T_c (approx. 42 °C) and the vesicle solutions were incubated at the various preheating temperatures indicated, prior to the mixing with the shift reagent.

Two phenomena can be observed. For incubation temperatures up to about 42 °C, the bilayer can be seen to remain permeable to Eu^{3+}, as manifested in the spectra by a single signal upfield-shifted for both the inside and outside choline methyls. For those samples preheated above T_c, the bilayer was no longer permeable to Eu^{3+}; only the signal which corresponds to the outside choline methyl protons is shifted to higher fields. It is obvious from Fig. 1 that an annealing-type process is

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* Certain lanthanide ions, such as Eu^{3+}, Pr^{3+} and Nd^{3+}, frequently referred to as shift reagents, have been shown to result in a chemical shift dispersion of the inside- and outside-facing choline methyl signals in the proton magnetic resonance spectrum without severe spectral broadening (see e.g. ref. 18), provided the paramagnetic shift reagent is added only either to the inner or outer compartment of the vesicles, but not to both, and the bilayer is impermeable to these ions. Such vesicles can be rendered isotonic, if a diamagnetic lanthanide ion, e.g., La^{3+}, is added to the other compartment. Phospholipid bilayer vesicles are considered to be impermeable to these cations under usual conditions. However, should the bilayer membrane be permeable to these cations, this ion permeability can be followed by monitoring the chemical shift difference between the two choline signals in the presence of one of these shift reagents either in the extra- or intravesicular medium.

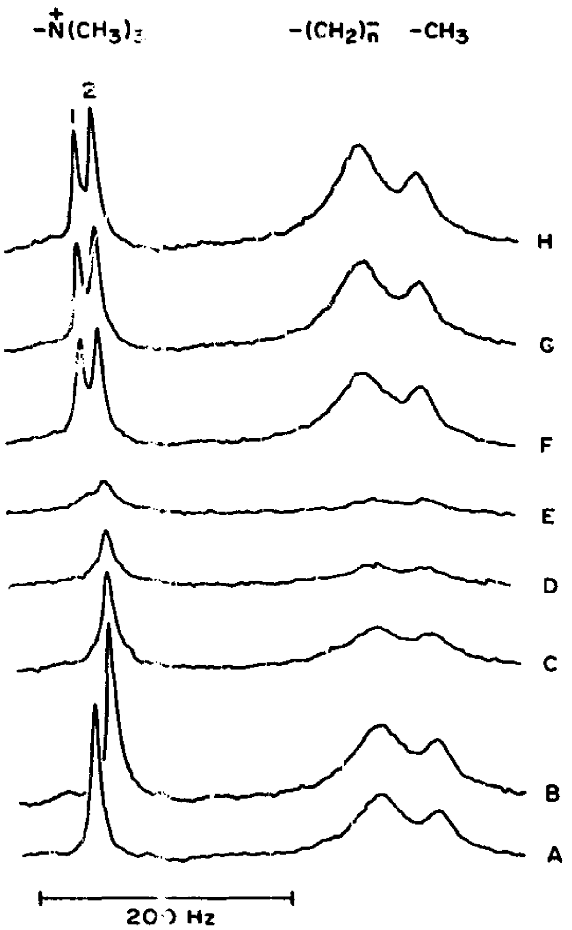

Fig. 1. Effect of annealing at various temperatures on the proton magnetic resonance spectra of dipalmitoyl phosphatidylcholine vesicles which contain structural defects. Purified dipalmitoyl phosphatidylcholine vesicles in ^2H_2O were initially prepared by sonication below room temperature in the presence of 5 mM $La(NO_3)_3$. Various samples were then incubated for 30 min at different temperatures. Immediately following the incubation, the samples were cooled to room temperature, and appropriate amounts of 5 mM $Eu(NO_3)_3$ solution ($La(NO_3)_3$ in the case of the control sample A) were added to them so that the final dipalmitoyl phosphatidylcholine concentration was 0.75 % w/v and the vesicles were isoosmotic. The proton magnetic resonance spectra of these vesicles were then recorded at 50 °C. The incubation temperatures were: A and B, 23 °C; C, 38 °C; D, 40 °C; E, 42 °C; F, 45.5 °C; G, 50 °C; and H, 55 °C. The spectral assignments indicated correspond to the choline methyl protons ($-N(CH_3)_3^+$) and the methylene and methyl protons of the hydrocarbon chains ($-(CH_2)_n-$, $-CH_3$). The two choline signals, denoted by 1 and 2, refer to the headgroups located on the inner and outer halves of the bilayer respectively.

taking place at high incubating temperatures ($T > T_c$) and that this annealing starts at the phase transition temperature. The second phenomenon demonstrated in Fig. 1 is vesicle fusion. The high resolution signal intensities for both the choline methyl as well as the fatty acid methylene and methyl protons show a pronounced minimum for incubating temperatures around T_c. The most plausible explanation for this loss in intensity in the high resolution nuclear magnetic resonance spectrum is vesicle fusion [2, 8]. A notable feature of our observations is that the widths of the lipid resonances are only affected to a minor degree even though a drastic intensity loss is being monitored. This very observation has previously been reported by Prestegard and Fellmeth [7] in their studies of the fusion of dimyristoyl phosphatidylcholine vesicles. Since a gradual increase in the vesicle size results primarily in gradual broadening of the lipid resonances without pronounced reduction of the observable proton magnetic resonance intensity until vesicles of a critical size (approx. 500 Å) are reached, these results indicate that the vesicle fusion process under study here is a multivesicular one, producing mainly vesicles which are larger than the critical size, at least under the conditions of the present experiments. The residual proton magnetic resonance intensity observed then, we surmise, arises from vesicles which have escaped the fusion. This conclusion is supported by electron microscopy studies.

Interrelationships between annealing and vesicle fusion

Further experiments revealed that this vesicle fusion is functionally related to the annealing of phospholipid vesicles, i.e., while unannealed vesicles tend to fuse at a

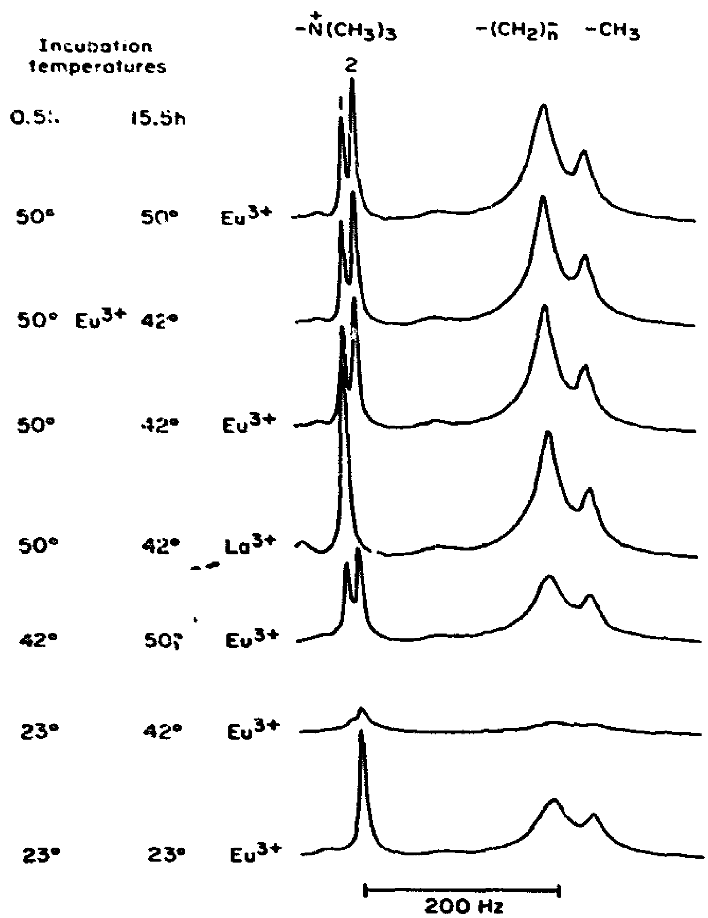

Fig. 2. Dependence of the proton magnetic resonance spectra of dipalmitoyl phosphatidylcholine vesicles which contain structural defects on the duration and temperature of annealing. Experimental details are given in text. (From the top the spectra are from samples A through G sequence.)

rapid rate, which seems to have a maximum around T_c, annealed vesicles are stable with respect to this fusion process. This result is demonstrated in Fig. 2. Here dipalmitoyl phosphatidylcholine vesicles were first prepared by sonication below T_c. We then incubated different samples of dipalmitoyl phosphatidylcholine vesicle solutions (all derived from the same stock solution) at different temperatures for 0.5 h. Samples A, B, C and D, 50 °C, sample E, 42 °C; samples F and G, 23 °C. After this initial brief incubation, the samples were again incubated for 15.5 h, either at the same or some other temperature. Of the samples annealed at 50 °C, one (sample A) was left at 50 °C (above T_c), while two samples (C and D) were incubated at 42 °C. Eu^{3+} was added to the remaining sample (sample B) prior to a second incubation at the phase transition temperature (42 °C). Sample E, which was preheated at 42 °C, was incubated at 50 °C (above T_c), while of the two samples which were left below T_c at 23 °C, one was incubated at the same temperature but the other at T_c. After this second heating, Eu^{3+} was added isotonically to the vesicle solutions (La^{3+} to the control sample D), and the samples were then annealed at the nuclear magnetic resonance measuring temperature (above T_c) in order to stop any further fusion and to fix the state of the vesicle dispersion prior to the recording of the proton magnetic resonance spectra. The final lipid concentration in all the samples was 1.5 % w/v.

It is obvious from the spectra presented in Fig. 2 that vesicle fusion, as manifested by the loss of high resolution signal intensity, depends entirely on the history of the vesicle suspension. Even after incubation for 15.5 h at 42 °C (T_c of dipalmitoyl phosphatidylcholine), the proton magnetic resonance spectra did not reveal evident changes for previously annealed samples (compare spectra for samples B, C and D with that for sample A), whereas the spectrum of a previously unannealed sample (F) suffered pronounced intensity loss during the second incubation period (compare samples A, B, C and D with sample F). The observed spectral differences between samples B, C and D vs. sample E reflect vesicle-vesicle fusion in sample E during the first incubation period as a result of the lower temperature of the initial annealing (42 °C instead of 50 °C). It is evident upon comparison between samples A and B that the vesicles are annealed after an 0.5 h incubation at 50 °C. Neither sample F nor G was annealed during the first incubation because of the low temperature of the incubation. Accordingly, extensive fusion of the vesicles occurred in sample F during the second prolonged incubation at 42 °C. For sample G, which was kept at 23 °C throughout these experiments, these vesicles stayed leaky towards Eu^{3+} even after 16 h of incubation at this temperature.

Structural differences between unannealed and annealed vesicles

We thus have evidence for some sort of structural defects within the bilayer structure when lecithin vesicles are prepared by sonication below the phase transition temperature of the lipid. These structural defects apparently can be annealed out simply by heating the vesicles above the thermal phase transition of the lipid. Although these defects have so far been inferred from the high permeability of the unannealed bilayer vesicles towards ions and the strong tendency of these vesicles to undergo fusion, we have, in fact, evidence for structural differences between annealed and unannealed bilayer samples below T_c.

Fig. 3 shows proton magnetic resonance spectra of a dipalmitoyl phosphatidylcholine sample before and after annealing (10 min at 52 °C) and recorded at 36.5 °C

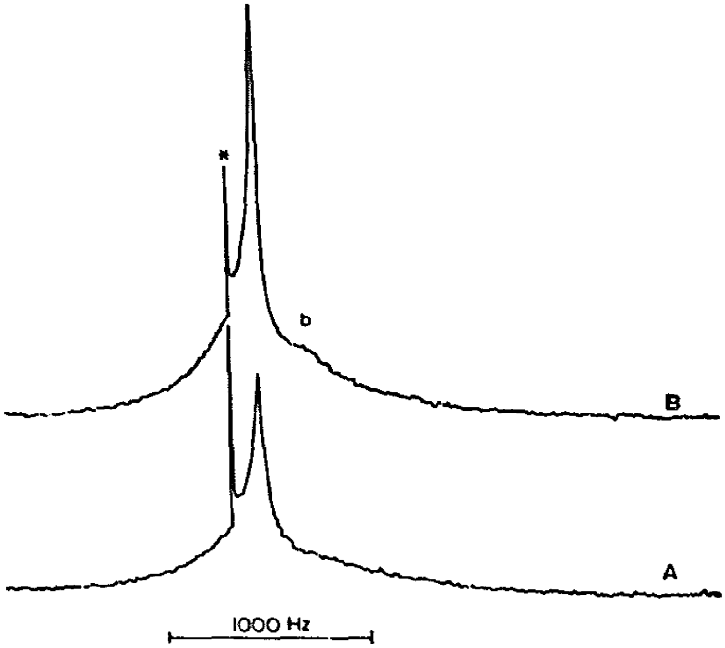

Fig. 3. Fourier transform ^1H nuclear magnetic resonance spectra (100 MHz) of the sonicated dipalmitoyl phosphatidylcholine solutions (2.5 w/v % in ^2H_2O containing 5 mM $La(NO_3)_3$). Sample B was the same as A except that it has been annealed for 10 min at 52 °C. Nuclear magnetic resonance measurements were made at 36.5 °C, which is below the phase transition temperature of dipalmitoyl phosphatidylcholine (approx. 42 °C). Peak b is the aliphatic methyl and/or methylene signals. *, residual HDO signal, which was partially saturated by the fast pulsing cycle.

(below T_c). Between the unannealed and the annealed sample we observed an approximate 50 % increase in the intensities together with a spectral sharpening of the hydrocarbon chain signal. Identical observations have also been obtained for distearoyl phosphatidylcholine. These results indicate that the packing of the bulk of the lipid molecules in unannealed bilayer vesicles is more regular or ordered than that in annealed vesicles below T_c. We surmise then that the high permeability of the unannealed structure towards ions is caused by imperfect grain boundaries between otherwise homogeneous and ordered lipid domains. Unannealed bilayer vesicles could also be permeable to Eu^{3+} because of a highly fluid, but homogeneous bilayer structure. This is in principle possible because the ideal hexagonal packing of the lipid molecules in multilayers must be modified to satisfy the "pseudo" hexagonal packing in vesicles with high surface curvatures. However, this possibility seems unlikely in light of the nuclear magnetic resonance data presented here, since such a bilayer membrane would be expected to yield sharper resonances before, rather than after, annealing, just the reverse of what is observed (Fig. 3). Electron microscopic studies also support the idea of cracks in the bilayer structure between well-ordered lipid domains. It has not been possible so far to obtain electron micrographs for unannealed phospholipid vesicles by the negative staining method, presumably because the staining solution leaks through these regions in the bilayer structure.

On the basis of these observations, we therefore conclude that the unannealed bilayer vesicle below T_c consists of a discontinuous arrangement of highly ordered lipid domains, with domain boundaries which are sufficiently structurally perturbed or disordered to serve as channels for rapid ion permeation. This conclusion, together with reasonable assumptions regarding the mechanism of formation of single-walled bilayer vesicles via the process of sonication, should permit us to define the nature of these structural defects more fully, and we shall attempt to do so later on in this paper.

Thermal stability of the structural defects

In order to define the nature of the structural defects more completely, we have measured the time-course of the thermal process by which these defects become annealed out. The kinetics of the annealing process are difficult to measure because at temperatures $\gg T_c$, the process is too rapid to be easily monitored by nuclear magnetic resonance methods, while at temperatures just above T_c, it becomes impossible to separate annealing from fusion. To partly circumvent this problem, we have, prior to the annealing measurements, allowed the vesicle solution to fuse for a definite time just below T_c, where annealing does not take place. Annealing studies were then conducted on the vesicle suspension after the vesicle fusion has subsided. The rationales behind this procedure are that (i) annealing of the structural defects is apparently very slow below T_c; and (ii) at a given temperature, the vesicle fusion rate slows down either with increasing vesicle size and/or with decreasing vesicle concentration. In addition, we have found that the fusion process itself does not automatically anneal previously unannealed vesicles.

In the following annealing studies, a 1.5% (w/v) dipalmitoyl phosphatidylcholine vesicle suspension was first prepared by sonication below T_c and allowed to sit for 60 min just below T_c. Using this vesicle suspension, we then carried out the following annealing experiments. Samples were incubated for various times at 3 °C above T_c, then cooled back to room temperature, and Eu^{3+} was added. After thorough mixing, the vesicles were annealed in order to fix their state. Spectra were then taken above T_c, and the quantity

$$\frac{\{I_L(t)/[I_L(t)+I_H(t)]\}}{\{I_L(\infty)/[I_L(\infty)+I_H(\infty)]\}}\times 100 = R\,(\%)$$

was then plotted vs. the incubation time. $I_L(t)$ refers to the intensity of low-field choline signal (not shifted by Eu^{3+}) after annealing time t, and $I_H(t)$, the intensity of the high-field choline methyl signal (shifted upfield by Eu^{3+}). Since $[I_L(t)+I_H(t)]$ should be independent of incubation time, the quantity R is equivalent to $I_L(t)/I_L(\infty)\times 100$, which can be readily seen to be related to the percentage of inward-facing phospholipid molecules in fully annealed vesicles, and hence may be taken as a measure of the extent of annealing.

Results of the above annealing experiments are presented in Fig. 4, where R is plotted versus the annealing time. Initially, i.e., at $t = 0$, the vesicles are completely permeable to the added Eu^{3+}, and the observed upfield shifted choline signal includes both the inside and outside facing choline resonances so that $I_L(0) = 0$. During this initial period, the annealing is in fact too fast to measure. Those vesicles which contain only a few defects no doubt contribute to this rapid annealing. With increasing incubation time, the low-field (unshifted) choline methyl signal starts to grow, its

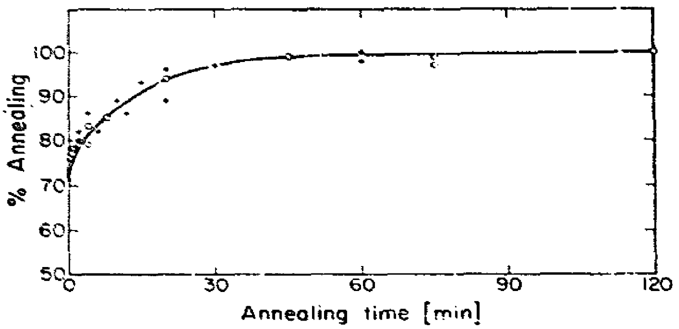

Fig. 4. The kinetics of annealing of dipalmitoyl phosphatidylcholine vesicles which contain structural defects. The quantity used to measure the extent of annealing is defined in the text. Annealing temperature, 45 °C; +, 1.5 % w/v dipalmitoyl phosphatidylcholine; ○, 0.5 % w/v dipalmitoyl phosphatidylcholine.

intensity reflecting the number of bilayer vesicles which have become fully annealed. Note that by our method of determination, vesicles are either fully annealed and thus impermeable to Eu^{3+} (also Nd^{3+}), or they are not totally annealed and are leaky to these ions. As the data in Fig. 4 indicate, annealing is complete within 1 h under these experimental conditions (3 °C above T_c). At higher temperatures, e.g., 10 °C above T_c, we have found that the annealing is completed within 10 min.

The rate of annealing of phospholipid vesicles is independent of the lipid concentration, so that the process is univesicular. This may be contrasted to the fusion process, which exhibits a complex dependence on the lipid concentration [7].

The fact that the annealing process takes about 1 h at 45 °C to be completed is somewhat surprising, because bilayer membranes of dipalmitoyl phosphatidylcholine are known to be quite "fluid" at these conditions. Since the lateral diffusion of lipid molecules within each monolayer is rapid above T_c [12], we suspect that the slower process associated with the annealing of the structural defects involved inside-outside migration of phospholipid molecules. This point will be discussed further in this paper.

The effect of salt

When dipalmitoyl phosphatidylcholine multilamellar dispersions are sonicated above T_c, one invariably obtains clear, bluish solutions of single-walled vesicles, irrespective of whether the solutions are salt-free or contain mono-, di-, or trivalent cations, or Cl^-, NO_3^-, $H_2PO_4^-$, CH_3COO^- as anions. When the sonication process was carried out below T_c, however, we found that the nature of the solution depended in an important way on the ionic environment, particularly on the charge of the cation (at constant ionic strength), but there was a conspicuous lack of dependence on the type of anions present. Trivalent cations seemingly favor the formation of single-walled vesicles; with decreasing cation charges there was a greater tendency for the formation of multi-walled vesicles. Since it is generally assumed that during sonication large multilayer units are broken down into small bilayer fragments which then reassemble to form single-walled or multi-walled vesicles of various sizes [13], we surmise that electrostatic interactions between these bilayer fragments could suppress the formation of multi-walled vesicles during the reassembly process, should these fragments become charged via the binding of a small number of trivalent or divalent cations. Aside from this fundamental difference between solutions containing trivalent cations and those with cations of lower charge, all bilayer vesicles prepared by sonication below T_c contain structural defects which could be annealed by heating above T_c, and which could promote vesicle fusion near T_c. Annealing experiments, where we replaced $Eu(NO_3)_3$ by $K_3Fe(CN)_6$ as the shift reagent, or Mn^{2+} to broaden the appropriate choline methyl signals showed the same behavior as what we had described earlier, thus the phenomena are not peculiar to the use of lanthanide ions, but are in fact general.

Mixed lipid systems

Evidence for structural defects in bilayer vesicles prepared by sonication below the lipid phase transition was not confined to single-component lipid systems. We summarize here some results which we have obtained for a number of two-component lipid mixtures.

(*i*) *The dipalmitoyl phosphatidylcholine-distearoyl phosphatidylcholine system.* Cosonication of mixtures of dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine at a dipalmitoyl phosphatidylcholine/distearoyl phosphatidylcholine weight ratio of 1 : 1 or 1 : 2 below room temperature and in the presence of La^{3+} gave homogeneous vesicle suspensions which exhibit random mixing of the two lipids, as indicated by the behavior of these vesicles towards annealing and annealing dependent fusion. The onset of annealing was found to start at 49 and 51 °C for the 1 : 1 and 1 : 2 mixtures, respectively. These temperatures correspond to the weighted mean of the T_c values for the pure lipids. Absorbance measurements of these vesicle suspensions also exhibit an abrupt break in the absorbance at these same temperatures.

(*ii*) *The dipalmitoyl phosphatidylcholine-cholesterol system.* When a 4 : 1 molar mixture of dipalmitoyl phosphatidylcholine and cholesterol were cosonicated around room temperature, these vesicles also exhibit structural defects, which could be annealed. As the data summarized in Fig. 5 show, annealing starts and fusion seems to reach a maximum at a temperature of between 38.5 and 42 °C. Absorbance versus temperature measurements of this sample also revealed a lipid phase boundary or "phase transition" in this temperature range.

We have carried out preliminary experiments with higher cholesterol concentrations. The question of whether the onset of annealing at various phospholipid/cholesterol concentration ratios can be used to monitor lipid phase separation and to map out the phase diagram of this system is under current investigation and these results will be reported in due course.

Cross-fusion between unannealed dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles

A further question is whether fusion occurs only between unannealed vesicles or occurs between unannealed and annealed vesicles as well. This question is somewhat intriguing because, if vesicles with structural defects can fuse with annealed vesicles or biological membranes, the leaky or unannealed vesicles will provide a useful method to incorporate extracellular lipids into cell membranes and exogenous materials into cells. A convenient way to demonstrate the fusion between unannealed and annealed vesicles is to use two kinds of vesicles composed of different lipids. We

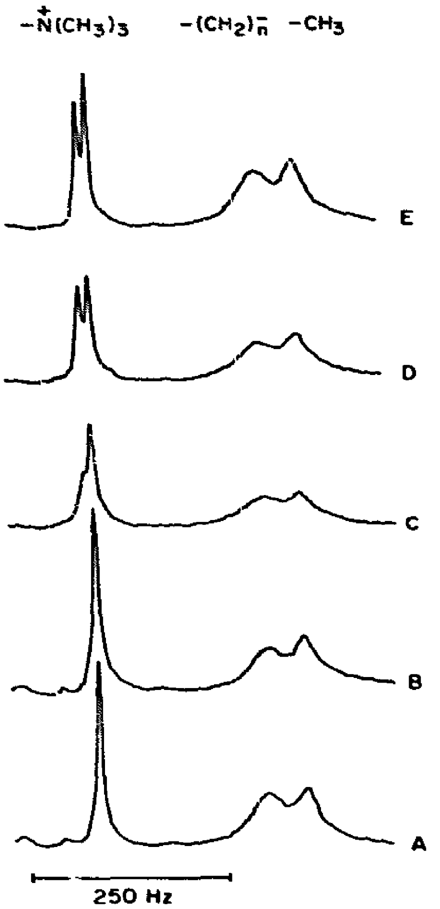

Fig. 5. Effect of annealing on the proton magnetic resonance spectra of dipalmitoyl phosphatidylcholine-cholesterol vesicles which contain structural defects. Dipalmitoyl phosphatidylcholine/cholesterol molar ratio: 4/1. These vesicles were prepared by cosonication of a 4 : 1 dipalmitoyl phosphatidylcholine/cholesterol mixture around room temperature in 5 mM $La(NO_3)_3$. Equal volumes of a 5 mM $Eu(NO_3)_3$ solution were added (at 23 °C) to a number of samples after they have been incubated for 0.5 h at the following temperatures: A, 23 °C; B, 38.5 °C; C, 42 °C; D, 46 °C; and E, 50 °C. All proton magnetic resonance spectra were recorded at 60 °C. Lipid concentration: 2.5 % w/v dipalmitoyl phosphatidylcholine.

used dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles with and without structural defects for this purpose. Any cross-fusion between these dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles should lead to fused vesicles with the total range of lipid compositions, as long as the fusion is monitored over a sufficiently long period of time. This is so, because the fusion process is expected to be essentially random and non-specific, provided the incubation temperature of the sample is sufficiently below the T_c values of the respective lipids, say around room temperature in the case of the present experiments. This heterogeneity in the lipid composition of the fused vesicles can be ascertained by monitoring the lipid phase transition of the vesicles as distearoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine form essentially regular solutions over the whole range of composition and the lipid phase transition for this system varies linearly with the composition.

In a series of experiments, separate suspensions of dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles were first prepared by sonication below their respective T_c values. Part of these stock solutions (2.5 % w/v in lipid with 5 mM $La(NO_3)_3$) were then annealed at appropriate temperatures. From these stock solutions, mixtures of dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles were prepared by mixing equal volumes of the two lipid solutions, either in their annealed or unannealed state. The four samples, corresponding to the four possible combinations of annealed and unannealed dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles, were then incubated at room temperature for 16 h. After this incubation period, all samples were annealed to fix their state and to stop further fusion, and their absorbances were recorded as a function of temperature. These results are summarized in Fig. 6.

In order to fully appreciate the significance of these results, we compare in

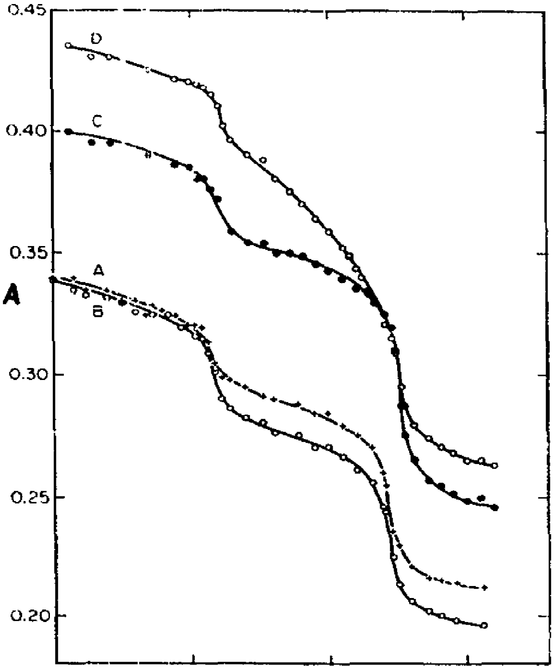

Fig. 6. Absorbance at $\lambda = 600$ nm vs. temperature curves obtained for four different 1 : 1 mixtures of dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles in $La(NO_3)_3$, after 16 h incubation at 23 °C and a 0.5 h annealing at 72 °C. Original state of the vesicle solutions were: (A) dipalmitoyl phosphatidylcholine (annealed)+distearoyl phosphatidylcholine (annealed). (B) Dipalmitoyl phosphatidylcholine (unannealed)+distearoyl phosphatidylcholine (annealed). (C) Dipalmitoyl phosphatidylcholine (annealed)+distearoyl phosphatidylcholine (unannealed). (D) Dipalmitoyl phosphatidylcholine (unannealed)+distearoyl phosphatidylcholine (unannealed). The total lipid concentration in each sample was 2.5 % w/v.

TABLE I

ABSORBANCES AT λ = 600 nm OF PURE AND MIXED DIPALMITOYL PHOSPHATIDYLCHOLINE/DISTEAROYL PHOSPHATIDYLCHOLINE VESICLES IMMEDIATELY AFTER PREPARATION AND AFTER A 15.5 h INCUBATION AT 23 °C

a, refers to annealed vesicles and n, unannealed vesicles.

	$t = 0$	$t = 15.5$ h
Dipalmitoyl phosphatidylcholine (a)	0.2	0.21
Dipalmitoyl phosphatidylcholine (n)	0.16	0.235
Distearoyl phosphatidylcholine (a)	0.51	0.51
Distearoyl phosphatidylcholine (n)	0.4	0.495
Dipalmitoyl phosphatidylcholine (a)+distearoyl phosphatidylcholine (a)	0.37	0.375
Dipalmitoyl phosphatidylcholine (a)+distearoyl phosphatidylcholine (n)	0.324	0.36
Dipalmitoyl phosphatidylcholine (n)+distearoyl phosphatidylcholine (a)	0.353	0.365
Dipalmitoyl phosphatidylcholine (n)+distearoyl phosphatidylcholine (n)	0.295	0.367

Table I the absorbances of pure dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles, both annealed and unannealed, as well as 2-component mixtures of these vesicles, taken immediately after preparation ($t = 0$) and after 15.5 h incubation at 23 °C. As we had mentioned earlier, there is essentially no change in the turbidity of the annealed samples over the 15.5 h period [11]. By contrast, the unannealed samples have lower absorbance values to start with, but they exhibited absorbance increases of approx. 0.07–0.1 units during the incubation. The observations for the mixed-lipid samples were particularly striking. For the mixtures where both types of lipid vesicles were annealed to begin with, there was hardly any change in the turbidity of the samples. For the two annealed-unannealed combinations, there was a small, but real, turbidity increase, and for the mixture made up of unannealed dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles, a pronounced increase in the turbidity was observed. For the system under consideration, the most reasonable interpretation for the turbidity increases is vesicle fusion. Thus, we attribute the small increase in the turbidity of the annealed-unannealed mixtures to self-fusion of the unannealed dipalmitoyl phosphatidylcholine or distearoyl phosphatidylcholine vesicles, and the more pronounced turbidity increase for the unannealed-unannealed sample to self-fusion of the unannealed dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles as well as to cross-fusion between them. This straightforward interpretation is supported by the temperature dependence of the absorbances for the various samples presented in Fig. 6. For three of these samples, namely, the annealed-annealed mixture and the two annealed-unannealed combinations, the absorbance data exhibit two sigmoidal breaks, at temperatures corresponding to the thermal phase transition of the two individual lipids (42 and 56 °C), together with a plateau in the absorbance vs. T plots between these limiting temperatures. The conclusion which one can draw from these results is that there is no detectable lipid transfer between dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles for these samples, and although vesicle fusion is indeed taking place in the case where one species of the lipid vesicle is unannealed, this vesicle fusion is confined to this one species. By contrast, when both species of lipid vesicles in the mixture were unannealed, cross-fusion between the two vesicle species occurs, as is evident by the pronounced turbidity increase after the long incubation and the continuous variation in the absorbance of the sample between the T_c values of the pure lipids.

Fusion between mixed lipid (unsaturated) vesicles [14] and heterofusion between saturated and unsaturated lecithin vesicles [5] have previously been reported. It is also generally believed that unsaturated lipids favour cell fusion [15]. However, these earlier observations must be contrasted with our present results, where the activation energy towards vesicle fusion is obviously significantly lowered by the presence of structural defects in the lipid bilayer. This structural-defect driven vesicle fusion is readily terminated by the annealing of the vesicle suspension.

Nature of structural defects and a model for the annealing process

In this section, we look into the question of how structural defects are produced within the bilayer structure by sonication below T_c of the lipid and propose a model for the annealing process. Any model which we propose for the unannealed vesicles must take into account, in addition to the data already mentioned so far, the fact that there is seemingly no measurable difference in the partial specific volume of the lipid between unannealed and annealed states below T_c, at least as determined for a 5 % dipalmitoyl phosphatidylcholine dispersion. This very result suggests that there exists only minor structural differences between annealed and unannealed vesicles. The situation here is therefore quite distinct from the rather large structural differences which have been noted between small sonicated vesicles and multilamellar dispersions, where an approximate 1 % difference in the apparent molal volumes has been reported below T_c [2].

On the other hand, the spectral intensity of the acyl chain proton signals increased by approx. 50 % upon annealing of a vesicle suspension which has been prepared by sonication below T_c (Fig. 3) [11]. This increase in spectral intensity can only be interpreted in terms of decreased motional order and/or increased segmental mobility of the lipid hydrocarbon chains upon annealing of the bilayer vesicles, with possibly an increase as well in the rate of lateral diffusion of the lipid molecules within the bilayer halves. In our judgment, then, we can rule out the possibility that the unannealed vesicles are leaky because of a looser packing of the lipid molecules within the bilayer structure, as the nuclear magnetic resonance observations reveal just the opposite behavior with annealing of these vesicles.

The most likely explanation for the behavior of unannealed vesicles is that they contain structural defects or dislocations between crystalline lipid domains, which permit rapid permeation of ions across the bilayer membrane. We surmise that heating of the vesicle solution to a temperature above the crystalline ⇌ liquid crystalline phase transition temperature leads to a sealing of these imperfections, a process which is undoubtedly facilitated by the higher mobility of the lipid molecules in the liquid crystalline state. If this is the case, the process of annealing should be an irreversible one, and indeed, we found that, once the vesicles have been annealed, rapid cooling of the vesicle suspension from temperatures well above T_c to temperatures well below T_c does not recreate leaky vesicles. These dislocations should also catalyze the fusion of small bilayer vesicles. Small sonicated vesicles are metastable because of their high excess surface free energy and tend to undergo vesicle-vesicle fusion once the opportunity is rendered. Although the activation energy for this fusion process is normally high so that the fusion is slow, we believe that it can be significantly lowered in the presence of these dislocations, particularly when the collision between two of these vesicles can bring the structural defects to meet. This simple picture thus not only accounts for the high permeability of unannealed bilayer vesicles towards ions, but also the high rate of fusion which we have observed for the unannealed vesicle suspensions.

The question remains as to how these dislocations are created in the first place and how they become annihilated by the thermal annealing process. We propose that dislocations within the bilayer structure are the result of a population defect in the distribution of the lipid molecules between the two monolayers. For a given vesicle size, the distribution of lipid molecules between the two sides of the bilayer are determined by geometrical constraints. Thus if the outer monolayer is somewhat deficient in lipid molecules or if the inside-facing monolayer contains a slight overpopulation, the packing of the lipid molecules can no longer be uniform throughout the bilayer structure and structural dislocations can result from the strain created. Although lateral diffusion of the lipid molecules can take place within each monolayer and is sufficiently rapid that one obtains, in the case of mixed lipid systems, a random distribution of the lipid molecules within each half of the bilayer, this process leaves the population defect unperturbed. A population defect can only be remedied by the outside $\rightleftharpoons$ inside lipid migration via the flip-flop mechanism, which would account for the long time constant which we have obtained for the annealing process [16, 17].

It is not difficult to understand why a population defect in the distribution of lipid molecules between the two monolayers is so readily formed by the sonication procedure. Finer [13] has previously argued that during sonication, large bilayer structures, either multilayers or single-walled vesicles, are broken down into small bilayer fragments, and these bilayer fragments then reassemble to form single-walled vesicles of various sizes. This fragmentation and reassembly process continues until the vesicles finally reach a limiting size (usually 250–300 Å in diameter), where presumably the lateral tension inherent in these highly curved bilayers prevents them from further fragmentation by shear forces. That this picture is substantially correct is indicated by the following experiments. (i) A short resonication (15 min at $T < T_c$) of small annealed dipalmitoyl phosphatidylcholine or distearoyl phosphatidylcholine lipid vesicles in the presence of Eu^{3+} in the extravesicular medium shows that Eu^{3+} became incorporated into the intravesicular compartment only for a small number of the vesicles. On the other hand, for large, annealed vesicles, nearly all the final vesicles (small) contain Eu^{3+} in their inner compartment after the second sonication. Insofar as we could determine, this corresponds to an all-or-none process, and we have detected no intermediate states where only a few Eu^{3+} have permeated the bilayer membrane, as these vesicles will give rise to a proton signal for the inner choline group at intermediate spectral positions. (ii) Resonication of a mixture of separately annealed small dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles gave no evidence for appreciable lipid transfer between the two species of lipid vesicles. However, when solutions of large annealed dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles were mixed the resultant vesicles after a second sonication were essentially homogeneous and had the same lipid composition as the random mixture.

Thus it appears that single-walled vesicles are formed by reassembly of small bilayer fragments. For thermodynamic reasons, these bilayer fragments are expected to have rather similar numbers of lipid molecules in the two monolayers. However, since a small sonicated vesicle 250–300 Å in diameter contains in excess of twice as many lipid molecules in the external monolayer than in the inside-facing half, the geometrical constraints on the assembly of a single-walled vesicle are serious, and it is not unreasonable that the finally assembled unit is usually formed with a population defect.

Finally, we point out that structural defects are not intrinsic of vesicle solutions which are produced by sonication below T_c, but are also found in vesicles prepared by the dialysis method, provided the dialysis is carried out below the lipid phase transition temperature. To demonstrate this, 2 % dipalmitoyl phosphatidylcholine (w/v) was dissolved in 2 % sodium cholate (w/v) and thoroughly mixed until a clear solution was obtained. This solution was then divided into two parts. One sample was dialyzed against a 10 mM Tris buffer (pH 7.4) at 50 °C to remove the cholate, and the other was dialyzed at 23 °C. $Eu(NO_3)_3$ was then added to both samples at room temperature and the proton magnetic resonance spectra taken at 50 °C. These nuclear magnetic resonance measurements revealed that the lipid dispersion obtained by dialysis at 23 °C contains structural defects which could be annealed by heating of the sample above T_c of dipalmitoyl phosphatidylcholine, while the vesicle dispersion dialyzed at 50 °C was devoid of such structural defects.

CONCLUSION

In this paper, evidence is presented for structural defects in phospholipid bilayer vesicles which are prepared by sonication below the crystalline ⇌ liquid crystalline phase transition of the lipid. These structural defects permit the rapid permeation of ions across the lipid bilayer and catalyze the rapid fusion of these vesicles. Heating the vesicle suspension above the lipid phase transition anneals out these structural defects and renders the vesicles impermeable to ions and stable towards fusion. It is proposed that these structural defects correspond to dislocations in the bilayer structure created by a population defect in the distribution of lipid molecules between inner and outer halves of the bilayer, which is formed during the initial assembly of a small bilayer vesicle. During the annealing process, presumably the population defect is corrected by an inside ⇌ outside lipid migration via the flip-flop mechanism.

The studies reported here suggest a rather simple procedure for the preparation of large single-walled bilayer vesicles. In principle, the conditions of fusion and annealing could be manipulated to optimize the production of vesicles within a given size range. The possibility of using the temperature corresponding to the onset of annealing as a means of mapping the liquidus phase boundary in the phase diagram for 2-component lipid mixtures also suggests itself. These potential applications of the present findings will be presented elsewhere in due course.

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