Fusion of Phosphatidylethanolamine-Containing Liposomes and Mechanism of the L_α–H_{II} Phase Transition†

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ABSTRACT: The initial kinetics of fusion and leakage of liposomes composed of N-methylated dioleoylphosphatidylethanolamine (DOPE-Me) have been correlated with the phase behavior of this lipid. Gagné et al. [Gagné, J., Stamatatos, L., Diacovo, T., Hui, S. W., Yeagle, P., & Silvius, J. (1985) *Biochemistry 24*, 4400–4408] have shown that this lipid is lamellar (L_α) below 20 °C, is hexagonal (H_{II}) above 70 °C, and shows isotropic ^{31}P NMR resonances at intermediate temperatures. This isotropic state is also characterized by complex morphological structures. We have prepared DOPE-Me liposomes at pH 9.5 and monitored the temperature dependence of the mixing of aqueous contents, leakage, and changes in light scattering upon reduction of the pH to 4.5. At and below 20 °C, where the lipid is in the L_α phase, there is very little aggregation or destabilization of the liposomes. Between 30 and 60 °C, i.e., where the lipid is in the isotropic state, the initial rates of liposome fusion (mixing of aqueous contents) and leakage increase. At temperatures approaching that where the hexagonal H_{II} phase transition occurs, the initial rates and extents of fusion decrease, whereas leakage is enhanced. Similar results were found for dioleoylphosphatidylethanolamine/dioleoylphosphatidylcholine (2:1) liposomes. These results clearly establish a common mechanism between the appearance of the isotropic state (between the L_α and H_{II} phases) and the promotion of liposome fusion. We propose a simple model to explain both the observed behavior of phosphatidylethanolamine-containing membranes with respect to liposome fusion and/or lysis and the beginning of the L_α–H_{II} phase transition.

It has long been speculated that the polymorphism accessible to many naturally occurring lipids, e.g., phosphatidylethanolamine (PE),[1] underlies some of the mechanisms of biological membrane fusion [see Cullis & de Kruijff (1979), Siegel (1984), and Verkleij (1984) for reviews]. These studies have focused primarily upon the equilibrium transition between the lamellar (L_α) phase to the hexagonal (H_{II}) phase. While it is obvious that complete membrane phase transitions to the H_{II} phase are not likely to be relevant to biological fusion, the membrane destabilization produced by the first steps of this transition is potentially quite important.

Our previous studies have investigated the correlation of the temperature at which a PE-containing membrane undergoes the L_α–H_{II} phase transition, denoted T_H, with the initial kinetics of liposome destabilization. Specifically, we monitored the fusion (mixing of aqueous contents), leakage, and lipid mixing from liposomes composed of pure PE's and PE's mixed with other lipids over temperature ranges which cover the T_H of the lipid mixtures (Bentz et al., 1985b; Ellens et al., 1986). We found that at and above the T_H of the lipids, there was a vast enhancement in the rates of leakage and lipid mixing; however, the mixing of contents, which is the biologically relevant event, was abolished. On the other hand, liposome–liposome contact was required to initiate the destabilization of the liposomes. That is, the transition of the lipid structure from the lamellar to the H_{II} phase began only after the two liposomes came into contact.

These studies left the relevance of PE's polymorphism to biological fusion at an even more speculative state. At the H_{II} phase transition temperature, the polymorphism does not promote fusion, with the required concomitant mixing of aqueous contents and membrane components. It could be that the bilayer destabilization promoted by the PE is sufficient to initiate the fusion event, with other biological membrane components repressing the complete collapse of membrane structure. Now, however, we have discovered for two types of PE-containing liposomes that there is a direct correlation between liposome fusion (mixing of aqueous contents) and the existence of a well-known, but little understood, intermediate "phase" between the L_α and the H_{II} phases.

Many lipid systems which exhibit classical thermodynamic L_α and H_{II} phases also exhibit other distinct morphological states between these two phases. This intermediate state has been characterized by cubic X-ray diffraction patterns, iso-

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[1] Abbreviations: PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DOPE-Me, N-methylated DOPE; EPE, egg phosphatidylethanolamine; TPE, phosphatidylethanolamine prepared from egg phosphatidylcholine by transesterification; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine prepared from TPE; PS, phosphatidylserine; PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; CHEMS, cholesteryl hemisuccinate; ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; DPX, *p*-xylenebis(pyridinium bromide); T_H, temperature range demarking the lamellar L_α–hexagonal H_{II} phase transition; T_c, temperature range demarking the lamellar gel L_β–liquid-crystalline L_α phase transition; T_I, temperature range of isotropic ^{31}P NMR resonances between the lamellar L_α and the hexagonal H_{II} phases which demarks the isotropic state; IMI, inverted micelle intermediate(s) or intermembrane intermediate(s); DSC, differential scanning calorimetry; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; MLV, multilamellar vesicle(s).

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tropic ^{31}P NMR signals, and/or lipidic particles/interbilayer attachment sites using freeze–fracture electron microscopy (Luzzati & Reiss-Husson, 1966; Cullis & de Kruijff, 1978; Lindblom et al., 1979; Verkleij et al., 1979, 1982; Larsson et al., 1980; Fontell, 1981; Hui et al., 1981b, 1983; Sen et al., 1981; Hui & Boni, 1982; Borovjagin et al., 1982; Tilcock et al., 1982; Williams et al., 1982; Boni & Hui, 1983; Verkleij, 1984; Rilfors et al., 1984; Gagné et al., 1985). For some surfactants, this intermediate state is a well-defined cubic phase, which also gives rise to isotropic ^{31}P NMR resonances (Rilfors et al., 1984). On the other hand, for the phospholipid mixtures studied, most of the electron microscopy of this state has shown the concomitant existence of lamella, small H_{II} tubes, and lipidic particles in a wide range of shapes and sizes. For these phospholipids, we shall use the term isotropic state to identify its operational definition, i.e., the morphological state(s) giving rise to isotropic ^{31}P NMR resonances. For reasons specified below, we believe that the isotropic state does not always represent a separate thermodynamic phase.

The studies on phospholipid mixtures agree on three aspects of the isotropic state: it is morphologically complex; it is very hysteretic (i.e., the thermal history of the lipid strongly affects the observed structures); and it mediates the L_α–H_{II} phase transition. They also speculate that it is important for some types of membrane fusion. The first two points have been well established, which explains the absence of a simple, coherent description of the isotropic state(s). The third point seems correct, but the heterogeneity of the intermediate morphologies has made modeling the event difficult. The fourth point, on the relevance of the isotropic state to membrane fusion (between two apposed membranes), is found by this study to have some basis in fact.

In order to examine this issue, we have chosen to use liposomes composed of DOPE-Me (N-methylated dioleoylphosphatidylethanolamine) and of DOPE/DOPC. In a sense, DOPE-Me is intermediate between DOPE and DOPC. Other methylated PE's have been studied with regard to elucidating the differences between PE and PC (Vaughan & Keough, 1974; Casal & Mantsch, 1983; Fuller et al., 1983; Mulukutla & Shipley, 1984). Recently, Gagné et al. (1985) found that DOPE-Me exhibits an isotropic state over a broad temperature range using ^{31}P NMR and freeze–fracture electron microscopy. This study makes DOPE-Me an excellent choice for examining the role of the isotropic state on liposome fusion.

Briefly, our results are that fusion of these liposomes occurs only within the temperature range of the isotropic state. At higher temperatures, this fusion is abolished as the liposomes undergo a contact-mediated lysis. After some period of fusion, there is a sudden collapse of the large aggregates of fusion products which results in a complete release of contents and, down to at least 10 °C below T_H, an enhanced fluorescence intensity of a lipid-bound NBD. This enhanced NBD fluorescence intensity has been shown to correlate extremely well with the formation of the H_{II} phase as measured by calorimetry and NMR. Thus, we believe that a significant portion of the lipid has achieved the H_{II} phase under these conditions. This would imply that the high-temperature regime of the isotropic state is simply the product of a kinetically hindered transition to the H_{II} phase. We propose that the metastability of these molecular intermediates of the isotropic state also causes the fusion of the apposed liposomes.

Materials and Methods

Dioleoylphosphatidylethanolamine (DOPE) with a single methyl substitution at the amino group (DOPE-Me), dioleoylphosphatidylcholine (DOPC), and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE prepared from transesterified egg PC (NBD-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). 1-Aminonaphthalene-3,6,8-trisulfonic acid (disodium salt) (ANTS) and *p*-xylenebis(pyridinium bromide) (DPX) were from Molecular Probes Inc. (Junction City, OR). The liposomes were prepared according to Szoka and Papahadjopoulos (1978). Further details on all these procedures can be found in Ellens et al. (1985, 1986).

For the ANTS/DPX leakage and fusion assay, the liposomes contained either (i) 25 mM ANTS and 45 mM NaCl, (ii) 90 mM DPX, or (iii) 12.5 mM ANTS, 45 mM DPX, and 22.5 mM NaCl. All solutions were buffered with 10 mM glycine at pH 9.5. In all cases, the encapsulated solutions were isoosmotic to the buffers used for the column chromatography and in the leakage and fusion experiments. There was no significant binding of ANTS to the exterior of the liposomes (Ellens et al., 1984, 1985, 1986). To measure the change in NBD fluorescence quantum efficiency, liposomes composed of DOPE-Me or DOPE/DOPC (2:1) containing 0.1 mol % of NBD-PE were made in the glycine buffer. The liposomes were extruded through polycarbonate membranes with 0.1-μm pores and separated from unencapsulated material on Sephadex G-75 (Pharmacia) using 100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA (pH 9.5) for the elution buffer.

The size distribution of the liposomes was measured by using dynamic light scattering (Coulter Model N4, Coulter Electronics, Inc., Hialeah, FL). With DOPE-Me, the *Z*-average diameter was 270 ± 95 nm for the ANTS liposomes, 220 ± 75 nm for the ANTS/DPX liposomes, and 280 ± 100 nm for the DPX liposomes. The encapsulated volume was 3.2 μL/μmol of lipid for the ANTS/DPX-containing liposomes and 5 μL/μmol of lipid for the ANTS-containing liposomes. With DOPE/DOPC (2:1), the *Z*-average diameter was 225 ± 80 nm for the ANTS liposomes, 200 ± 65 nm for the ANTS/DPX liposomes, and 205 ± 65 nm for the DPX liposomes. The encapsulated volume was 2.6 μL/μmol for the ANTS/DPX liposomes and 5.4 μL/μmol for the ANTS liposomes. Since the area per DOPE-Me head group in the bilayer is probably in the range of 40–60 Å^2 (Mulukutta & Shipley, 1984), we know that some of the liposomes have two or more lamellas. The absolute rates of leakage and fusion varied between liposome preparations, but the temperature dependence of the kinetics was the same for all liposome preparations. The delay times of collapse (see Figure 1C) varied between preparations but always decreased with increasing temperature and increasing liposome concentration. The encapsulated contents of liposomes stored at pH 9.5 and 4 °C showed no measurable leakage for at least a week.

Fluorescence and light scattering were measured in an SLM 4000 fluorometer (SLM Instruments, Champaign—Urbana, IL) equipped with two 90° emission channels, allowing both fluorescence and light scattering to be monitored simultaneously. Leakage is measured with liposomes containing both ANTS and DPX (Ellens et al., 1984). With the ANTS/DPX fusion assay, mixing of aqueous contents of ANTS- and DPX-containing liposomes is registered as a decrease in ANTS fluorescence due to quenching of ANTS by DPX (Ellens et al., 1985). The fluorescence scale is calibrated with the fluorescence of a 1:9 mixture of ANTS and DPX liposomes in glycine buffer (100 mM NaCl, 2 mM glycine, and 0.1 mM EDTA, pH 9.5) taken as 100% fluorescence (0% fusion). The 0% fluorescence level was set with buffer. Excitation was at 360 nm and emission >530 nm. On the figures, fusion (% max *Q*) is equal to 100 minus the recorded fluorescence, which is equal to the percentage of ANTS which is quenched by DPX

at that time. ANTS fluorescence is not sensitive to pH between 9.5 and 4.5, and the quenching efficiency of DPX is not affected by pH in this region. The incubations were started by injection of 25 μL of a 2 M sodium acetate/acetic acid buffer, pH 4.5, into a magnetically stirred cuvette, containing 1 mL of the liposome suspension in glycine buffer (100 mM NaCl, 2 mM glycine, and 0.1 mM EDTA, pH 9.5).

We used the increase in quantum efficiency of NBD-PE, incorporated at 0.1 mol % in DOPE-Me or DOPE/DOPC (2:1) liposomes, to measure the onset of the hexagonal phase transition (Ellens et al., 1986; Baldwin et al., 1986). The H^+-induced increase in quantum efficiency of NBD-PE was measured at various temperatures as described previously (Ellens et al., 1986). The fluorescence of the 0.1% NBD-PE liposomes at pH 9.5 was set to 100%, and the 0% level was set with buffer. We recorded NBD fluorescence as a function of time upon lowering the pH to 4.5. Excitation was at 450 nm and emission >530 nm.

For calorimetry, DOPE-Me or DOPE/DOPC (2:1) was dispersed in glycine buffer (2 mM glycine, 100 mM NaCl, and 0.1 mM EDTA, pH 9.5) at a concentration of 180 μmol/mL. The lipid dispersion was sonicated for 1 h under argon in a bath-type sonicator. The liposomes were then dialyzed overnight (at 4 °C) against three changes of pH 4.5 acetate buffer (50 mM acetate/acetic acid and 100 mM NaCl, pH 4.5); 0.7 mL of the liposome suspension, containing 125 μmol of total lipid, was used for the thermograms with a Microcal MC-1 high-sensitivity differential scanning calorimeter. A scan rate of 35 °C/h was used. As a check, we measured the L_α–H_{II} phase transition temperature range for 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) at pH 4.5 and found it to be 71–75 °C, in excellent agreement with Epand (1985). For DOPE-Me, our results agree with Gagné et al. (1985). For the DOPE-Me and DOPE/DOPC (2:1) systems, we found that the L_α–H_{II} phase transitions always occurred at the temperatures specified in Table I, but that the line shape of the thermogram varied from run to run. Our estimate for the enthalpy of the L_α–H_{II} phase transition is less than 0.1 kcal/mol in both cases, and we believe the variability is due to instrumental sensitivity limits.

Results and Discussion

Figure 1 shows the temperature dependence of the fusion of the DOPE-Me liposomes induced by H^+ at pH 4.5 where the PE is zwitterionic. The mixing of aqueous contents is monitored by the ANTS/DPX fusion assay, as described under Materials and Methods. Panel A shows the fusion curve for the temperature range of 20–45 °C. At 20 °C, Gagné et al. (1985) find that the lipid is completely lamellar, and we see that the rate of fusion is very slow, ~1%/min. At 30 °C, there is a small isotropic component in the ^{31}P NMR signal, but at 40 °C, DOPE-Me clearly shows both lamellar and isotropic ^{31}P NMR signals (Gagné et al., 1985). We see that the rate of fusion increases in this temperature range. Thus, the onset of the isotropic signal demarks the beginning of significant liposome fusion.

Panel B shows the fusion curves for the temperature range of 50–80 °C. Gagné et al. (1985) find a pure isotropic ^{31}P NMR signal at 50 and 60 °C and an H_{II} phase signal at 80 °C. By DSC, the T_H is measured at 68–77 °C. The fusion signals respond very strongly to these temperature ranges. From 40 to 60 °C, there is an increase in the initial rates of fusion, and the extents of mixing of contents are about the same. At 65 °C, the extent of fusion starts decreasing substantially. At 80 °C, the end of the T_H, the fusion is essentially

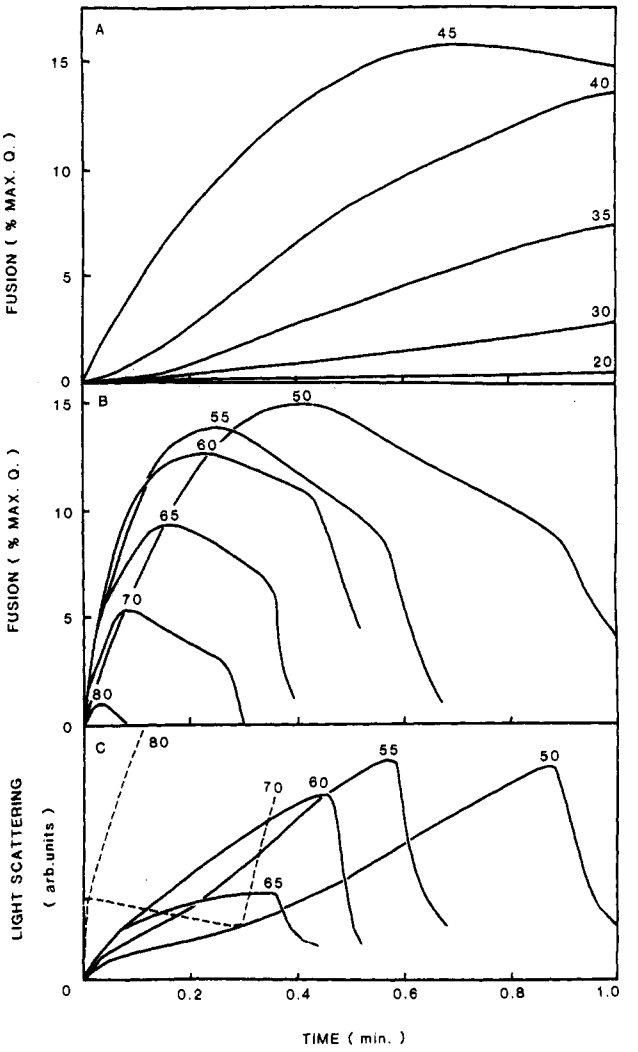

FIGURE 1: H^+-induced fusion (mixing of aqueous contents) of DOPE-Me liposomes at various temperatures. At $t = 0$, the pH of a 50 μM 1:9 mixture of ANTS- and DPX-containing liposomes was lowered from pH 9.5 to 4.5. Note that the fusion curves are given in the units of percent maximum quenching, i.e., 100 minus the measured percent maximum fluorescence signal. Panel A shows the fusion curves between 20 and 45 °C and panel B between 50 and 80 °C. Panel C shows the 90° light-scattering signals between 50 and 80 °C, measured simultaneously with the fusion. At and above T_H, i.e., 70 °C, the light-scattering signal *increases* after the collapse. These data are shown by the dashed lines in Figure 1C.

abolished. Thus, fusion is promoted in the temperature ranges of the isotropic ^{31}P NMR signal. Furthermore, the extent of fusion starts decreasing at temperatures where the H_{II} phase emerges, in agreement with our previous work with pure PE's and their mixtures with other lipids (Bentz et al., 1985b; Ellens et al., 1986).

After the fusion curves reach a maximum, the signals slowly decline due to leakage from the fused liposomes. Leakage like this has been observed in many cases of liposome fusion, e.g., Ca^{2+} and PS (Wilschut et al., 1983; Bentz & Düzgüneş, 1985) and pure PE liposomes (Ellens et al., 1986). The feature of the fusion curves in panel B, which has *never* been observed with other fusing systems, is that after some time delay the rate of leakage from the fused liposomes is sharply enhanced. The liposomal contents are rapidly ejected into the medium. In panel C, we show the corresponding 90° light-scattering signals for the fusion curves of panel B. Clearly, at the same times as the rate of leakage from the fused liposomes increases, there is a precipitous change in the scattered light intensity:

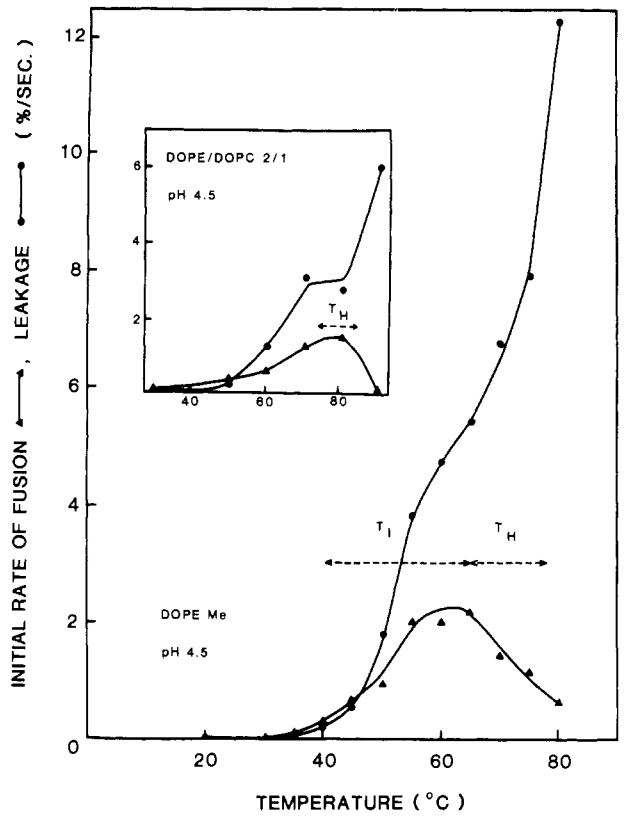

FIGURE 2: Initial rates of H^+-induced fusion and leakage of DOPE-Me liposomes as a function of temperature. The initial rates are just the initial slopes of the fusion curves shown in Figure 1 and the leakage curves (data not shown) in units of percent per second. The inset shows the temperature dependence of the initial rates of fusion and leakage of DOPE/DOPC (2:1) liposomes at 125 μM total lipid concentration.

a decrease below T_H and an increase above T_H. There is no simple and rigorous explanation for the reversal in the direction of the intensity change. Nonetheless, the effect is consistent, and it demarks a collapse of the aggregates of fused liposomes. Below we will describe evidence that this collapse also signals that a significant portion of the lipid has achieved the H_{II} phase.

We performed the same experiments using liposomes composed of DOPE/DOPC (2:1) in order to examine the specific role of the surface density of the *N*-methyl groups. Tilcock et al. (1982) have shown that DOPC coexists with DOPE in the H_{II} phase. Thus, we might expect the DOPE/DOPC (2:1) mixture to present the same *average* surface as DOPE-Me in both the L_α and H_{II} phases. The kinetic behavior of the liposomes is quite similar, differing only in a higher temperature requirement for the DOPE/DOPC (2:1) system.

Figure 2 shows the initial rates of both fusion and leakage as a function of temperature. The inset shows the same data for the DOPE/DOPC (2:1) liposomes. For DOPE-Me, the temperature range of the isotropic ^{31}P NMR signal is demarked by T_I, while T_H denotes the DSC-measured H_{II} transition range from Gagné et al. (1985). Our DSC measurements for DOPE-Me gave the same T_H range as Gagné et al. (1985), and for DOPE/DOPC (2:1), we found $T_H \sim$ 75–85 °C, as shown in Table I and indicated on the inset to Figure 2. While DOPE/DOPC (2:1) does not yield an isotropic ^{31}P NMR signal *below* 40 °C (Tilcock et al., 1982), our kinetic data imply that it would be found, but only *above* 40 °C. Isotropic ^{31}P NMR resonances have been found for DOPE/DOPC (1:1) from 50 to 90 °C (Cullis et al., 1978) and for DOPE/DOPC (4:1) at 40 °C (Tilcock et al., 1982).[2]

Table I: L_α–H_{II} Phase Transition Temperatures

lipid	cation	T_H (°C) DSC	T_H (°C) NBD[c]
DOPE-Me	pH 7.4/200 mM NaCl	68–77[a]	
	pH 4.5/100 mM NaCl	68–76[b]	64–71
DOPE/DOPC (2:1)	pH 4.5/100 mM NaCl	75–85[b]	80–87

[a] Gagné et al. (1985). [b] From this work. [c] From this work. The range is given for initial rates of NBD fluorescence increases of 0.1–1.0%/s from Figure 3.

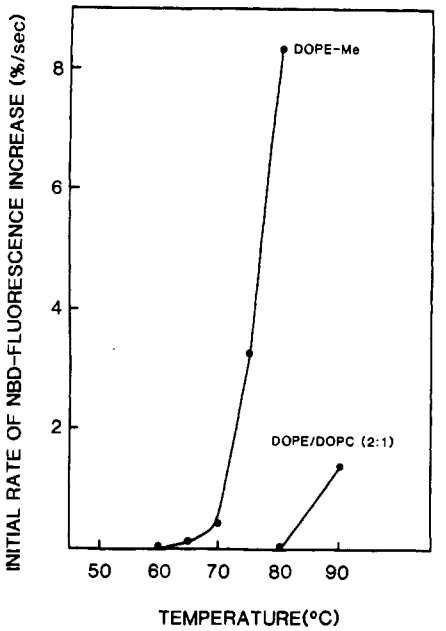

FIGURE 3: Effect of temperature on the fluorescence of DOPE-Me and DOPE/DOPC (2:1) liposomes containing 0.1 mol % NBD-PE. DOPE-Me and DOPE/DOPC (2:1) liposomes (50 and 125 μM, respectively) were incubated in pH 9.5 glycine buffer (2 mM glycine, 100 mM NaCl, and 0.1 mM EDTA) at various temperatures. At t = 0, the pH was lowered to 4.5 by injection of 25 μL of a 2 M acetate/acetic acid buffer, pH 4.5. The increase in fluorescence was recorded as a function of time. The fluorescence scale was calibrated with the fluorescence of the 0.1 mol % NBD liposomes at t = 0 as 100% and buffer as 0%. The initial rate of NBD fluorescence increase above the 100% level was plotted as a function of temperature (Ellens et al., 1986).

The initial rates of fusion for DOPE-Me begin increasing at the beginning of T_I and start decreasing in the T_H temperature range. For DOPE/DOPC (2:1), we can only say that the initial rates of fusion increase above 40 °C and decrease as the temperature reaches T_H.

We were intrigued by the observation that the fusing liposomes would suddenly collapse after a certain time period. Examining this point, we found that this time period before collapse decreased with increasing temperature, as shown in Figure 1, and with increasing lipid concentration (data not shown).

To elucidate whether this collapse signaled a significant change in the lipid phase, we made liposomes containing 0.1 mol % NBD-PE. In Ellens et al. (1986) and Baldwin et al. (1986), it has been shown that the quantum efficiency of this

[2] Eriksson et al. (1985) have produced a very careful phase diagram for DOPE/DOPC mixtures, *in 10% (w/w) H_2O*, using ^{31}P NMR and ^2H NMR. For comparable systems, they find phase transitions occurring 10–30 °C below those found by Cullis et al. (1978), Tilcock et al. (1982), and our DSC data, all of which are taken in excess water, i.e., 80–95% (w/w) H_2O. This lowering of phase transition temperatures by dehydration is well-known: even egg PC can be found in the H_{II} phase at very low water contents (Luzzati et al., 1968).

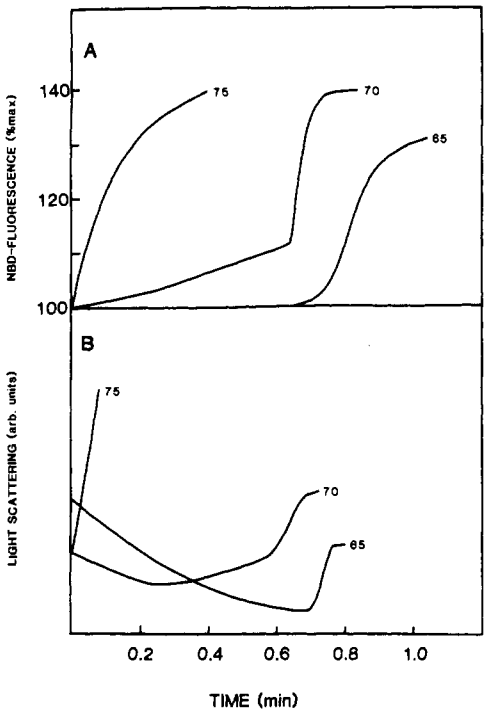

FIGURE 4: Time course of NBD fluorescence increase of DOPE-Me liposomes containing 0.1 mol % NBD-PE. (A) 50 μM DOPE-Me liposomes were incubated in pH 9.5 glycine buffer (2 mM glycine, 100 mM NaCl, and 0.1 mM EDTA) at 65, 70, and 75 °C as indicated by the numbers next to the curves. At t = 0, the pH was lowered to 4.5 by injection of 25 μL of a 2 M acetate/acetic acid buffer, pH 4.5. The fluorescence scale was calibrated with the fluorescence of the 0.1 mol % NBD liposomes at t = 0 as 100% and buffer as 0%. The increase in fluorescence was recorded as a function of time. Fluorescence and light scattering were monitored simultaneously, and the lower panel (B) shows the corresponding light-scattering traces.

lipid-bound fluorophore is higher in the H_{II} phase than in the L_α phase for several PE's, as well as with cardiolipin–Ca^{2+}. When the lipid remains in the L_α phase, the NBD fluorescence does not change, regardless of whether the liposomes are fusing or not.

In Figure 3, we show the initial rate of change of NBD fluorescence as a function of temperature for both DOPE-Me and DOPE/DOPC (2:1). The temperature range where these initial rates increase corresponds very well to the DSC-measured T_H range (Ellens et al., 1986), as shown also in Table I.

In Figure 4, we show the fluorescence and the 90° light scattering over time for the NBD-containing DOPE-Me liposomes at several temperatures. It is quite clear down to 60 °C that the collapse, as shown by the light scattering as in Figure 1, also signals a rapid increase in the NBD fluorescence. While this NBD assay is not quantitative for the extent of H_{II} phase formation, it is obvious from comparing the extents of fluorescence enhancement that a significant portion of the lipid has completed the transition to the H_{II} phase. It is important to note here that the NBD assay is the only available method for monitoring the formation of the H_{II} phase at these very low lipid concentrations, e.g., $\leq$100 μM.

The collapse of the aggregates is a very hysteretic event. Comparing Figures 1 and 4 shows that the time period to collapse differed for the fusion liposomes, e.g., 5 μM ANTS-containing liposomes and 45 μM DPX-containing liposomes, and 50 μM NBD liposomes. The leakage liposomes, 50 μM ANTS/DPX-containing liposomes, gave different times also. Furthermore, the direction of the change in the light-scattering signal can be variable when the temperature is very near the onset of the T_H range; see 65 °C. The collapse signals a change in both the scattering volume and the lipid refractive index, and the extent of these changes depends upon the aggregation kinetics in ways that we cannot yet rigorously explain.

Our results show that the fusogenic capacity of these liposomes can be predicted from a thermodynamic phase diagram. We have relied upon the results of Gagné et al. (1985) to supply some of this phase diagram and the temperature range of the isotropic state. There must be no confusion about the validity of this approach, especially as regards the hysteresis of the isotropic state. For DOPE-Me, below 20 °C the lipid is completely L_α; above 75 °C, the lipid is completely H_{II}, and this will be true regardless of sample preparation.[3] The hysteresis of the isotropic state may affect the relative intensity of the isotropic ^{31}P NMR resonance, at a given temperature, but not its existence. Since fusion experiments are best done at low lipid concentrations and NMR or calorimetric experiments require high lipid concentrations, there cannot be exact correspondence in sample preparation.

Figure 5 shows a representation of the molecular events which best summarize these data. The close apposition of the liposomes is magnified at step A, which shows just the apposed sections of the bilayers. When the temperature is below T_I [or below T_H for those PE's which show no isotropic state, e.g., EPE (Tilcock et al., 1984)], the equilibrium structure for pure PE's is lamellar sheets (Verkleij, 1984). We have found leakage, lipid mixing, and limited fusion for DOPE, EPE, and TPE liposomes under this condition (Ellens et al., 1986). Pryor et al. (1985) found lipid mixing with DMPE under this condition, but leakage and fusion were not examined. The molecular mechanism for this fusion is not known, although in Ellens et al. (1986) we explained why it is likely to be the same as that which produces fusion of phosphatidylserine (PS) liposomes at low pH.

When the temperature is above T_I, or above T_H for those phospholipids which have a facile H_{II} phase transition without the intervening isotropic state, the apposed bilayers can be connected initially by a few nonbilayer intermediate structures (shown in step B) which could be inverted micelles (Verkleij et al., 1979; Sen et al., 1981; Verkleij, 1984; Siegel, 1984, 1986) or other molecular architectures (Miller, 1980; Hui et al., 1981a, 1983; Rand et al., 1981). For our purposes, the precise architecture of the nonbilayer intermediate structures is not crucial to the development of the model. Hence, we will use the more generic title of intermembrane intermediate, denoted IMI in the figure.

As shown in step B, the initial few IMI are expected to show reversibility, allowing reversion to apposed bilayers (Siegel, 1984, 1986). However, one can also expect that the existence of even a few IMI between the apposed bilayers would sharply decrease the reversibility of liposomal aggregation (Bentz et al., 1985a). We have examined this effect with DOPE/

[3] There is one important caveat to this statement. Cullis et al. (1978) have shown that when DOPE/DOPC (1:1) is hydrated at or below 30 °C, the lipid is in the L_α phase. At 90 °C, the lipid shows pure isotropic ^{31}P NMR resonances. However, when this lipid is cooled from 90 °C back to 30 °C, it still shows isotropic ^{31}P NMR resonances. Even as low as 4 °C, the isotropic state is stable for at least 4 days. When this lipid was cooled to −70 °C, i.e., below its T_c, and then reheated to 30 °C, its phase was again pure L_α. It would appear that the only expedient way to return an isotropic state lipid to a pure L_α phase is to reduce the temperature below its T_c. All of our lipid samples were prepared at or below 20 °C and stored at 4 °C until used for the kinetic or DSC studies. Since Gagné et al. (1985) followed essentially the same procedure, we can use their phase diagram for the DOPE-Me.

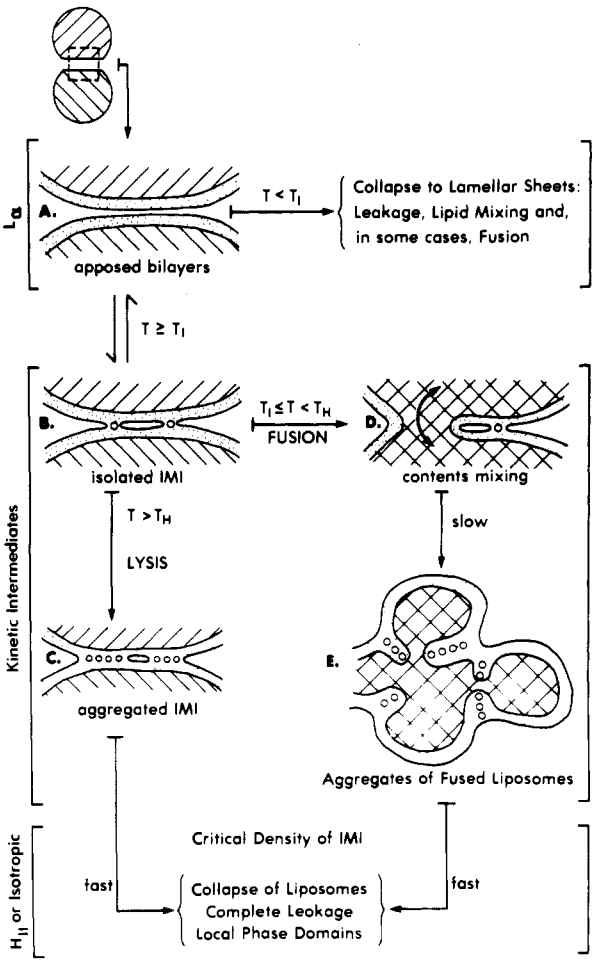

FIGURE 5: Representation of the fusion and destabilization pathways for liposomes composed of polymorphic lipids. Part of this scheme is adapted from Siegel (1984). Briefly, the apposed bilayers are shown in step A. When the temperature is below T_I, i.e., the lipids always exist in a pure L_α phase, these liposomes can in some cases show fusion, with mixing of aqueous contents, and will eventually collapse to the equilibrium phase of lamellar sheets. This mechanism is similar to Ca^{2+}- or H^+-induced fusion of PS liposomes. When the temperature exceeds T_I, or T_H for those phospholipids which show a facile H_{II} phase transition without the formation of the isotropic state, then a new mechanism of membrane destabilization appears which begins (step B) with the reversible formation of a few IMI, intermembrane intermediates, which are also the proposed initial molecular intermediates of the L_α–H_{II} phase transition. If the temperature is, in fact, above that of the calorimetrically determined transition temperature (T_H), then these IMI rapidly assemble (step C) into the configuration leading to facile formation of the H_{II} phase tubes. The liposomes undergo a contact-mediated lysis. On the other hand, if the temperature lies between T_I and T_H, i.e., where ^{31}P NMR shows isotropic resonances, and the lipid supports the isotropic state, then the liposomes show fusion, with the mixing of aqueous contents (step D). We believe that the isotropic state demarks the situation where isolated IMI have time to transform into fusion structures. In time, the large aggregates of fused liposomes (step E) suffer a precipitous collapse leading to immediate ejection of contents and (down to 10 °C below T_H) fluorometric evidence of substantial H_{II} phase formation. This collapse time is probably the time required for the aggregation–fusion products to become sufficiently large to accommodate enough IMI to promote an L_α–H_{II} phase transition with part of the lipid within the fused aggregate. A complete description is given in the text.

cholesteryl hemisuccinate liposomes (Bentz et al., 1985c).

When the temperature is above T_H, we propose that the number of IMI rapidly increases as shown at step C (Siegel, 1984, 1986). The accumulation of these IMI leads to rapid collapse of the apposed liposomes toward H_{II} phase structures, accompanied by leakage, lipid mixing, and *no* fusion (mixing of aqueous contents). Appropriately, we call this the *lytic* pathway. After complete collapse, the equilibrium H_{II} phase is achieved.

On the other hand, if the system exhibits isotropic ^{31}P NMR resonances and the temperature is in the T_I range, then IMI have sufficiently long lifetimes to produce membrane fusion by some other process (step D). Calculations by Siegel (1986) show that inverted micelle IMI can form new structures resulting in membrane fusion, but only if the IMI remain isolated. The correlation between the T_I and fusion would imply that this is a temperature range where not many IMI form initially. They have time to transform to fusion structures before a sufficient number of IMI can assemble (step E) and drive a collapse process into the final lipid product, the nature of which will be discussed below. According to Siegel (1986), the existence of long-lived inverted micelle IMI between the apposed bilayers of the MLV dispersions is consistent with the thermal and temporal hysteresis found in the high lipid concentration NMR, X-ray, and electron microscopic experiments on the isotropic state (Cullis et al., 1978; Hui et al., 1981, 1983; Tilcock et al., 1982; Boni & Hui, 1983; Gagné et al., 1985).

The eventual collapse of the liposomes after a time delay is compelling evidence that the delay of IMI accumulation is the reason for fusion being correlated with T_I (Figure 1B,C). The model of Siegel (1984, 1986) predicts that in contact areas between two ca. 0.2–0.3 μM sized liposomes at temperatures around T_I, there may be as few as one IMI. Since the first step in assembly of the H_{II} phase is aggregation of IMI between pairs of apposed bilayers, the lipid in such vesicles cannot begin to form the H_{II} phase rapidly, if at all. The lag time before collapse is probably the time required for aggregated liposomes to fuse into membranes big enough to permit the formation of enough IMI in the bilayer–bilayer interfaces to catalyze rapid H_{II} phase formation. It is obvious that rapid H_{II} phase formation would produce a precipitous leakage from the aggregated liposomes and a sudden end to further fusion, as is observed (Figure 1). The delay times in Figure 1C decrease with increasing temperature and lipid concentrations (data not shown). This is presumably due to the increase with temperature of both liposome aggregation rates (Bentz et al., 1985a) and the number of IMI per unit area of bilayer–bilayer interface (Siegel, 1984, 1986).

We have speculated here that after the observed collapse of the aggregates, much of the lipid should be in an H_{II} phase configuration, even at temperatures below the DSC-measured T_H. For these lipids, this may imply that the isotropic state is the result of a slow L_α–H_{II} phase transition with metastable molecular intermediates. We have used the NBD assay to show that the collapse is coincident with some H_{II} phase formation. With DOPE-Me liposomes, we found that there was an enhanced NBD fluorescence down to 60 °C occurring precisely *during* the collapse of the aggregates (data not shown). Below 60 °C, where light scattering still shows the collapse, the change in the NBD signal following collapse was not sufficiently large to reach any firm conclusions. However, Gagné et al. (1985) found some H_{II} tubes in DOPE-Me at 50 °C with freeze–fracture electron microscopy. The NBD assay for H_{II} phase formation is not quantitative, but it clearly shows that after the collapse at the higher temperatures a significant portion of the lipid is in a proper H_{II} phase configuration.

Our designation of T_I and T_H has been operational up to this point. Our data suggest that the temperature of the L_α–H_{II} phase transition for DOPE-Me may be as low as 30 °C, where the isotropic ^{31}P NMR resonance first appears, and that by 75 °C the transition rate for the majority of the lipid is fast enough to be measured calorimetrically. It is clear that

careful definition of terms will be required to avoid confusion on the rates of phase transitions with metastable intermediates vs. the thermodynamic requirements of phase behavior, e.g., the Gibbs phase rule.

It is evident that being able to slow the overall kinetics of the L_α–H_{II} phase transition, by limiting the area of apposed bilayer contact using low liposome concentrations, will be quite useful for elucidating its molecular mechanism (Siegel, 1984, 1985; Kirk et al., 1984; Gruner, 1985; Caffrey, 1985). There are at least two well-studied membrane systems whose fusion kinetics suggest a potential role for the isotropic state. The first is cardiolipin/PC, which has shown a Ca^{2+}-dependent ^{31}P NMR isotropic signal (de Kruijff et al., 1982) and liposome fusion rates which strongly depend upon both Ca^{2+} concentration and temperature (Wilschut et al., 1985). The second is the inner mitochondrial membrane which, in vitro, has been shown to undergo Ca^{2+}-induced fusion only at elevated temperatures and the extent of fusion shows a maximum as a function of the Ca^{2+} concentration (Chazotte et al., 1985).

It is worth emphasizing that liposome fusion may not occur for all lipids showing isotropic states. The isotropic state is, after all, a mixture of morphological structures, and some "types" of isotropic states may not promote fusion of aqueous contents. As we have argued above, a very critical parameter for fusion is the time for which the IMI can remain isolated. Membrane components which decrease this time will inhibit fusion, while enhancing the rate of lipid collapse into the H_{II} phase. This study, together with previous work, establishes that the relevance of the polymorphism accessible to PE-containing membranes to biological fusion resides primarily in the isotropic state and not the H_{II} phase, per se.

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