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Articles

Destabilization of Phosphatidylethanolamine-Containing Liposomes: Hexagonal Phase and Asymmetric Membranes†

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ABSTRACT: We have measured the temperature of the L_α–H_{II} phase transition, T_H, for several types of phosphatidylethanolamine (PE), their binary mixtures, and several PE/cholesteryl hemisuccinate (CHEMS) mixtures. We have shown for liposomes composed of pure PE and in mixtures with CHEMS that there is an aggregation-mediated destabilization which is greatly enhanced at and above T_H. We now ask the question: How well can a dioleoylphosphatidylethanolamine/CHEMS liposome, for example, destabilize TPE (transesterified from egg phosphatidylcholine)/CHEMS liposome and vice versa? We use Ca^{2+} and H^+ to induce aggregation and to provide different values of T_H: the T_H of the PE/CHEMS mixture is much lower at low pH than with Ca^{2+}. We find that if the temperature is above the T_H of one lipid mixture, e.g., A, and below the T_H of the other lipid mixture, e.g., B, then the destabilization sequence [measured by the fluorescent 1-aminonaphthalene-3,6,8-trisulfonic acid/*p*-xylylenebis(pyridinium bromide) leakage assay] is AA > AB ≫ BB. That is, the bilayer of the lipid A (which on its own would end up in the H_{II} phase) destabilizes itself better than it destabilizes the bilayer of lipid B (which on its own would remain in the L_α phase). The BB contact is the least unstable. From these experiments, we conclude that the enhanced destabilization of membranes provided by the polymorphism accessible to these lipids above T_H is effective even if only one of the apposed outer monolayers is H_{II} phase competent. The surprising result is that if the temperature is above the T_H of both lipid mixtures, then the destabilization sequence is AB > AA, BB. That is, the mixed bilayers are destabilized more by contact than either of the pure pairs. We believe that this is due to specific differences in the kinetics of aggregation or close approach of the membranes. Similar results were obtained with pure PE liposomes induced to aggregate by Ca^{2+} at pH 9.5. We also found that the kinetics of low-pH-induced leakage from PE/CHEMS liposomes were initially faster when the CHEMS on both sides of the bilayer is fully protonated. However, in a citrate buffer, which cannot cross intact membranes, the leakage was eventually faster. Flip-flop of the protonated CHEMS to the inner monolayer can explain this observation.

The ability of many naturally occurring lipids to undergo a bilayer L_α to hexagonal H_{II} phase transition has led to much speculation about the putative roles of this polymorphism in cell function [for reviews, see Cullis & de Kruijff (1979), Verkleij (1984), Siegel (1984, 1987a), Rilfors et al. (1984), Cullis et al. (1985), Gruner et al. (1985), Lindblom et al. (1986), Weislander et al. (1986), and Quinn et al. (1986)]. For the L_α–H_{II} phase transition to be relevant for biological membrane fusion, it must satisfy three criteria: (i) it must occur after the contact of the two membranes; (ii) it must result in the mixing of aqueous contents between the membrane-bound compartments; and (iii) it must function between

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asymmetric membranes, as that is the most common biological situation.

To the first point, it is known that multilamellar lipid dispersions can complete the L_α–H_{II} phase transition rapidly (van Venetië & Verkleij, 1981; Caffrey, 1985). We have recently shown that pure phosphatidylethanolamine (PE)[1] and PE-containing liposomes will begin the L_α–H_{II} transition immediately after liposomal contact (Bentz et al., 1985b; Ellens et al., 1986a). These studies also showed that the fusion, i.e., mixing of aqueous contents, of these liposomes found at lower temperatures was abolished as the temperature reached that of the calorimetrically measured L_α–H_{II} phase transition, T_H. At and above T_H, there is contact-mediated liposome lysis. Clearly, the L_α–H_{II} phase transition, taken to completion, is antithetical to biological fusion.

Nevertheless, with regard to the second point, the basic mechanism of the L_α–H_{II} transition can lead to fusion (with mixing of aqueous contents) when the molecular intermediates of the transition become stable. We have found examples of this using PE-containing liposomes (e.g., DOPE-Me, monomethylated dioleoylphosphatidylethanolamine or DOPE/DOPC) whose lipids showed isotropic ^{31}P NMR resonances at temperatures intermediate between those where the pure L_α and the pure H_{II} phases are found (Ellens et al., 1986b). In this temperature range, the liposomes showed mixing of aqueous contents, and as the temperature approached T_H, where the equilibrium H_{II} phase is rapidly formed, this fusion was abolished. It has been proposed that in this isotropic state the intermembrane attachments which begin the L_α–H_{II} phase transition are kinetically blocked from completing this transition and, instead, transform to structures which promote isotropic ^{31}P NMR resonances and liposome fusion, i.e., mixing of aqueous contents (Ellens et al., 1986b; Siegel, 1986b, 1987a,b).

In this study, we address the third point, i.e., the relationship of the L_α–H_{II} phase transition to the destabilization of asymmetric membranes. It is understood that the molecular compositions of two biological membranes subject to fusion will differ from one another, as also will the inner and outer monolayers of each membrane (Zwaal et al., 1975; Altstiel & Branton, 1983; Crabb & Jackson, 1985; Davey et al., 1985). Here, we have examined whether one bilayer, whose lipids would be in the H_{II} phase at equilibrium, can enhance the destabilization of another bilayer, whose lipids would be in the L_α phase at equilibrium.

A second question is how inside–outside asymmetry, i.e., where the outside monolayers are above their T_H and the inside monolayers are not, affects the kinetics of destabilization. To examine this asymmetry, we use two differently composed liposomes wherein a pH gradient between their inner and outer monolayers induces an L_α phase composition on the inside and an H_{II} phase composition on the outside. This asymmetry is different from that normally construed for biological membranes, e.g., amino and acidic phospholipids on the cytoplasmic surface and choline lipids on the extracellular surface. However, by using this pH gradient, we have made liposomes, for the time course of the experiment, which have a functional asymmetry; i.e., the outer monolayer is poised to begin the formation of the H_{II} phase, while its inner monolayer, as well as the entire bilayer of the other liposome, is stable in the L_α phase.

Briefly, the answer to these questions is that when the lipids are above their equilibrium T_H, the liposomes can be destabilized by contact with many lipid surfaces, and, in turn, they can destabilize some bilayers. They also can be very sensitive to inside–outside asymmetry. Thus, the lipid polymorphism implicit in the L_α–H_{II} phase transition satisfies the third basic criterion for relevance to biological fusion. Resolving these experimental data with the theoretical models of the L_α–H_{II} phase transition (Siegel, 1984, 1986a,b, 1987a,b; Kirk et al., 1984; Gruner, 1985; Caffrey, 1985; Fontell et al., 1985; Tate & Gruner, 1987) will considerably sharpen our understanding of its molecular mechanism.

Materials and Methods

Dioleoylphosphatidylethanolamine (DOPE), egg phosphatidylethanolamine (EPE), phosphatidylethanolamine prepared by transesterification from egg phosphatidylcholine (TPE), egg phosphatidylcholine (PC), and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) prepared from TPE were purchased from Avanti Polar Lipids (Birmingham, AL). CHEMS (cholesteryl hemisuccinate) was purchased from Sigma (St. Louis, MO). 1-Aminonaphthalene-3,6,8-trisulfonic acid (disodium salt) (ANTS) and *p*-xylylenebis(pyridinium bromide) (DPX) were from Molecular Probes Inc. (Junction City, OR).

The TPE/CHEMS (2:1), PC/CHEMS (2:1), and DOPE/CHEMS (2:1) liposomes were prepared according to Szoka and Papahadjopoulos (1978). Briefly, 15 μmol of lipid was dissolved in 1 mL of ether (stored over distilled, deionized water) and sonicated for 5 min in a bath-type sonicator under argon with 0.33 mL of the aqueous phase [a solution of either (i) 12.5 mM ANTS, 45 mM DPX, and 20.5 mM NaCl buffered with 10 mM Tris-HCl at pH 7.5 or (ii) 10 mM Tris-HCl and 100 mM NaCl, pH 7.5]. The resulting emulsion was evaporated in a rotary evaporator at room temperature under reduced pressure. After collapse of the gel, 0.66 mL of the aqueous phase was added, and the liposome suspension was then maintained under high vacuum (water aspirator) for 45 min to remove residual ether. These liposomes, denoted LUV, were extruded through polycarbonate membranes with 0.1-μm pores (Olson et al., 1979).

The pure PE liposomes, e.g., DOPE, EPE, or TPE, were prepared by hydration of 25 μmol of the lipid with 1 mL of either (i) 12.5 mM ANTS, 45 mM DPX, 22.5 mM NaCl, 0.1 mM EDTA, and 10 mM glycine, pH 9.5, or (ii) 10 mM glycine, 0.1 mM EDTA, and 100 mM NaCl, pH 9.5. The multilamellar liposomes were subsequently extruded through polycarbonate membranes with 0.1-μm pores (Olson et al., 1979). In Ellens et al. (1986a), we found that pure PE liposomes made by the REV procedure and extruded through 0.1-μm polycarbonate filters were oligomeric. The encapsulated volumes of the PE MLV extruded through 0.1-μm filters

[1] Abbreviations: PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; EPE, egg phosphatidylethanolamine; TPE, phosphatidylethanolamine prepared from egg phosphatidylcholine by transesterification; PS, phosphatidylserine; PC, egg phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine prepared from TPE; Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine prepared from dioleoylphosphatidylethanolamine; CHEMS, cholesteryl hemisuccinate; ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; DPX, *p*-xylylenebis(pyridinium bromide); EDTA, ethylenediaminetetraacetic acid disodium salt; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MLV, multilamellar vesicle(s); LUV, vesicles prepared by the reverse-phase evaporation method and extruded through a 0.1-μm filter; SUV, small unilamellar vesicle(s); T_H, temperature demarking the transition to the hexagonal H_{II} phase as measured calorimetrically; T_c, temperature of the onset of the lamellar gel–liquid-crystalline phase transition; T_I, temperature of the onset of the isotropic state between the L_α and H_{II} phases, demarked by isotropic ^{31}P NMR resonances; IMI, inverted micelle intermediate(s) or intermembrane intermediate(s); Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; DSC, differential scanning calorimetry; Dnp, dinitrophenyl; Cap, caproyl.

were essentially identical with those made by the REV procedure (see below).

The liposomes were separated from unencapsulated material on Sephadex G-75 (Pharmacia) by using either (i) 2 mM His, 2 mM Tes, 1 mM EDTA, and 100 mM NaCl, pH 7.4 (for the PE/CHEMS liposomes), or (ii) 10 mM glycine, 0.1 mM EDTA, and 100 mM NaCl, pH 9.5 (for the pure PE liposomes), as the elution buffer. In all cases, the encapsulated solutions were isoosmotic to the buffers used for the column chromatography and in the leakage experiments (see below). Liposomal lipid concentrations were determined by phosphate analysis (Bartlett, 1959).

The encapsulated volumes of the ANTS/DPX-containing DOPE/CHEMS and TPE/CHEMS liposomes were 2.0 and 2.6 μL/μmol, respectively. The encapsulated volume of the ANTS/DPX-containing liposomes composed of pure DOPE, EPE, or TPE was 9.4, 3.9, and 2.3 μL/μmol, respectively. We have determined previously (Ellens et al., 1984, 1985, 1986a) that there is no significant binding of ANTS to PE/CHEMS or pure PE liposomes.

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). The liposomes initially containing both 12.5 mM ANTS and 45 mM DPX emit ~4% of the fluorescence of the lysed liposomes, and this fluorescence is set to 0% leakage, while the fluorescence of the liposomes lysed in the appropriate buffer (using Triton X-100) is set to 100% leakage. Excitation was at 360 nm and emission >530 nm. Fluorescence intensities are insignificantly affected by changes in scattered light.

Ca^{2+}- and H^+-induced leakages were measured at various temperatures. The incubations with Ca^{2+} were started by injection of small volumes of $CaCl_2$ into a magnetically stirred cuvette, containing 1 mL of the liposome suspension in the appropriate buffer. The incubations at low pH were started by injection of small volumes of concentrated liposome suspensions into a magnetically stirred cuvette, containing 1 mL of the appropriate buffer (50 mM acetate/acetic acid and 66 mM NaCl, pH 4.5, or 50 mM citrate/citric acid and 55 mM NaCl, pH 4.5). In all cases, the buffers were made isoosmotic to the encapsulated contents of the liposomes using NaCl, and the osmolality was measured by using freezing point depression (Advanced Instruments Inc., Needham Heights, MA). The results were recorded on an Omniscribe chart recorder at high chart speeds when necessary.

We used the increase in quantum efficiency of NBD-PE, incorporated at 0.1 mol % in PE-containing bilayers, to measure the onset of the hexagonal phase transition (Ellens et al., 1986a,b; Baldwin et al., 1986) of the liposomes. The NBD assay for H_{II} phase formation is indirect; however, it rigorously meets two important criteria. First, for all the lipid systems we have examined, there is about a 50% increase in NBD quantum efficiency when the lipid transforms to the H_{II} phase [see Ellens et al. (1986a) for full curves]. We use the initial kinetics of that change as a convenient means to estimate the onset temperature of that transition. A rate of change of fluorescence in the range of 0.1–1.0%/s demarks a temperature range which contains or is at least within 10 °C of the calorimetric or ^{31}P NMR determined T_H, as shown in Table I. Such a correlation is quite sufficient for our purposes since we only use the NBD data to make sure that we are well below or above the T_H, i.e., at least 10 °C. Second, the NBD assay is currently unique in its ability to monitor lipid phase behavior under dilute lipid concentrations and, especially, between two apposed liposomes. The enhanced NBD fluorescence does not necessarily mean that the two apposed liposomes have collapsed to an H_{II} phase tube. However, it is clear that this quantum efficiency does respond to the molecular intermediates of the L_α–H_{II} phase transition which are accessible to two apposed liposomes.

For the PE/CHEMS mixtures, the NBD-containing liposomes were made according to Szoka and Papahadjopoulos (1978) with 10 mM Tris-HCl and 100 mM NaCl, pH 7.5, as the aqueous phase. The liposomes were extruded through polycarbonate membranes with 0.1-μm pores. For the pure PE liposomes and the PE mixtures, the lipids were hydrated in 10 mM glycine, 0.1 mM EDTA, and 100 mM NaCl, pH 9.5, and the resulting MLV dispersion was not extruded. We have found that the method of liposome preparation does not affect the transition temperature significantly.

The Ca^{2+}- and H^+-induced increase in the quantum efficiency of NBD-PE was measured at various temperatures as described previously (Ellens et al., 1986a). The fluorescence of the 0.1% NBD-PE liposomes in the absence of Ca^{2+} and either at pH 7.5 (PE/CHEMS) or at pH 9.5 (pure PE) was set to 100%, and the 0% level was set with buffer. We recorded NBD fluorescence as a function of time upon the addition of Ca^{2+} or lowering the pH to 4.5. Excitation was at 450 nm, and emission was measured at 530 nm.

Results

Our first step is to determine the temperature range (T_H) over which the lipids used here achieve the hexagonal H_{II} phase. In Ellens et al. (1986a), we showed that the T_H of DOPE, EPE, and TPE could be established by an increase in the quantum efficiency of NBD-PE fluorescence, incorporated at 0.1 mol % in the liposomes. Stubbs et al. (1987) found similar results and extended the validity of the assay to other H_{II} phase competent lipids. The assay functions either with liposomes made by the REV procedure or simply with vortexed MLV. Aggregation and lipid mixing below T_H leave the NBD fluorescence intensity largely unchanged. Above T_H, the increase in quantum efficiency of the NBD-PE leads to an increase in NBD fluorescence intensity over time as more of the lipid undergoes the transition.

In Figure 1, we have plotted this effect as the initial rate of the increase of NBD fluorescence as a function of temperature for a wide range of pure PE and PE/CHEMS liposomes. Empirically, we have found for several types of pure PE that initial rates between 0.1 and 1.0%/s correspond to the *onset* of the H_{II} phase transition as monitored by differential scanning calorimetry (DSC) (Ellens et al., 1986a,b). The T_H of DOPE has been determined by ^{31}P NMR and electron microscopy (van Dijck et al., 1976; Cullis & de Kruijff, 1978b; Tilcock et al., 1982) and recently by calorimetry using high-sensitivity DSC techniques (Epand, 1985; Gagné et al., 1985). The NBD assay agrees quite well with these techniques. For PE/CHEMS liposomes, the rate of increase of quantum efficiency is less with H^+- than it is with Ca^{2+}-induced phase transitions. One reason for this may be that protonated CHEMS, like cholesterol, broadens the temperature range of coexistence of the L_α and H_{II} phases (Lai et al., 1985a), although this broadening reflects the kinetics of the transition to some extent. The L_α–H_{II} phase transition for DOPE/CHEMS has only been determined by ^{31}P NMR (see Table I).

In Figure 1A, the pure PE's, as well as their binary mixtures, are examined at pH 9.5 when 20 mM Ca^{2+} is injected into

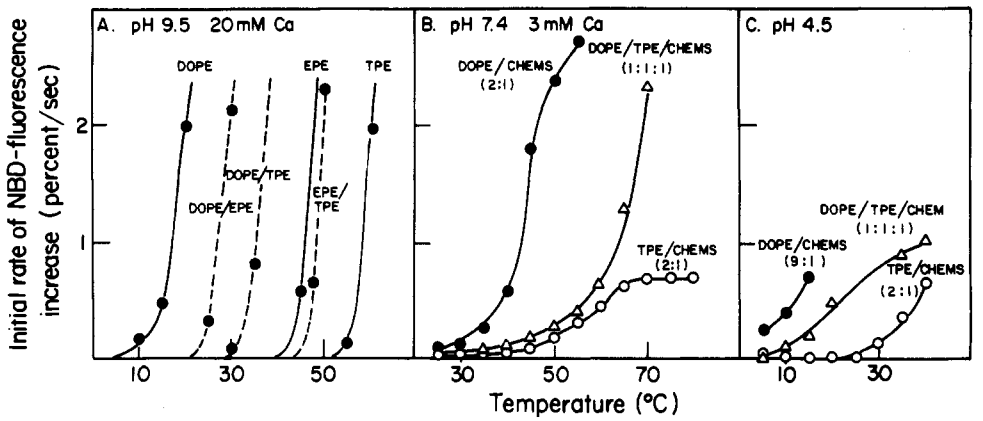

FIGURE 1: Initial rates of the Ca^{2+}- and H^+-induced increase in the fluorescence of 0.1 mol % NBD containing PE and PE/CHEMS liposomes at temperatures around T_H. The increase in fluorescence is due to the transformation of the lipid to the H_{II} phase and an enhanced quantum efficiency of NBD fluorescence in this phase. Panel A shows the initial rate of the fluorescence change induced by 20 mM Ca^{2+} added to PE MLV made at pH 9.5. The liposomes are composed of either pure DOPE, EPE, or TPE, as denoted by the solid lines, or of a 1:1 mixture of DOPE/EPE, DOPE/TPE, or EPE/TPE, as denoted by the dashed lines. Panel B shows similar data for PE/CHEMS LUV made at pH 7.4 when 3 mM Ca^{2+} is added. The LUV are composed of DOPE/CHEMS (2:1), DOPE/TPE/CHEMS (1:1:1), and TPE/CHEMS (2:1). For reasons explained in the text, we believe that the NBD fluorescence intensity increase below 70 °C for the TPE/CHEMS (2:1) in 3 mM Ca^{2+} *cannot* be ascribed to H_{II} phase formation. Panel C shows similar data for PE/CHEMS LUV made at pH 9.5 and incubated at pH 4.5. The LUV are composed of DOPE/CHEMS (9:1), DOPE/TPE/CHEMS (1:1:1), and TPE/CHEMS (2:1). For all cases, the fluorescence scale was set with the fluorescence of the buffer as 0% and that of the liposomes before the addition of the Ca^{2+} or H^+ as 100% fluorescence. The initial rates are just the initial slopes of the increase in fluorescence in the units of percent per second. As we have shown in Table I, and in Ellens et al. (1986a), this assay is relatively insensitive to the liposome type, i.e., whether LUV, extruded MLV, or just hand-shaken MLV are used.

the liposome suspension. These liposomes are prepared by vortexing an MLV dispersion. As mentioned, the NBD fluorescence increase is not strongly dependent upon the type of liposome used. Basically, the NBD assay would predict that the T_H for each of the binary mixtures, e.g., DOPE/EPE, is about halfway between that of the two pure PE's, i.e., DOPE and EPE. This shows that the lipids are miscible and that the T_H of the mixture is essentially just an average of the respective T_H's. This behavior has been found before for other PE mixtures (van Dijck et al., 1976; Tilcock & Cullis, 1982).

In Figure 1B, we show the behavior of DOPE/CHEMS (2:1), TPE/CHEMS (2:1), and their stoichiometric mixture DOPE/TPE/CHEMS (1:1:1) in 3 mM Ca^{2+} at pH 7.4. Here the liposomes are made by the REV procedure and extruded through 0.1-μm filters. At pH 7.4, these liposomes remain dispersed due to the net negative charge on CHEMS, which is protonated at ~pH 5.5 (Ellens et al., 1984). The behavior of the TPE/CHEMS (2:1) liposomes is anomalous in that the initial increase in fluorescence, at 50 °C, ceases at 70 °C. We also observed that the extents of fluorescence increase in this case are small (<20%) relative to the other cases [≥50%; see also Ellens et al. (1986a)]. These data strongly imply that this relatively small increase in fluorescence is not due to H_{II} phase formation. We know that with Ca^{2+}, the T_H of the PE/CHEMS mixture is higher than that of the pure PE (Lai et al., 1985a). Figure 1A shows TPE to have a T_H of about 55 °C. For TPE/CHEMS (7:3) in 3 mM Ca^{2+}, Lai et al. (1985a) observed H_{II} phase above 75 °C using ^{31}P NMR. While the NBD assay is quite convenient and unique as a means of monitoring H_{II} phase formation at low lipid concentrations, it must be recalled that other factors can affect the quantum efficiency of NBD (Silvius & Gagné, 1984a,b; Morris et al., 1985). For the present, we interpret these results to imply that the T_H of TPE/CHEMS (2:1) in 3 mM Ca^{2+} exceeds 75 °C.

In Figure 1C, we show the data for TPE/CHEMS (2:1), DOPE/TPE/CHEMS (1:1:1), and DOPE/CHEMS (9:1) at pH 4.5, where the PE's are zwitterionic and the CHEMS is protonated. The T_H of DOPE/CHEMS (2:1) at pH 4.5 is below 0 °C (Lai, 1985). In that study, it was shown that the T_H of DOPE or TPE was lowered by the addition of CHEMS at low pH and raised by the addition of CHEMS in Ca^{2+} at pH 9.5. The behavior in low pH *with* Ca^{2+} is not known, except that H^+ and Ca^{2+} will compete for the CHEMS binding site (Ellens et al., 1985). The NBD fluorescence does not

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

lipid	cations[a]	T_H (°C) NBD assay[b]	T_H (°C) other
DOPE	pH 7.4	–	5–15[c,d]
	pH 4.5	10–25[e]	–
	pH 9.5/20 mM Ca^{2+}	8–13[e]	–
EPE	pH 7.4	–	28–35,[f] 32–45,[g] 28[h]
	pH 4.5	40–45[e]	43–47[e]
	pH 9.5/20 mM Ca^{2+}	40–45[e]	45–50[e]
TPE	pH 7.4	–	63,[g] 50[h]
	pH 4.5	50–57	56–59,[e] 60[i]
	pH 9.5/20 mM Ca^{2+}	50–57	56–62[e]
DOPE/EPE (1:1)	pH 9.5/20 mM Ca^{2+}	20–27	–
DOPE/TPE (1:1)	pH 9.5/20 mM Ca^{2+}	30–35	–
EPE/TPE (1:1)	pH 9.5/20 mM Ca^{2+}	45–50	–
DOPE/CHEMS			
9:1	pH 7.4	–	>60[i]
	pH 4.5	<5	<0[i]
2:1	pH 7.4	–	>60[i]
	pH 4.5	<0	–
	pH 7.4/3 mM Ca^{2+}	30–40	30–37[i]
TPE/CHEMS			
2:1	pH 7.4	–	>60[i]
	pH 4.5	~30	10–18[i]
	pH 7.4/3 mM Ca^{2+}	<75	~75[i]
7:3	pH 4.5	–	23–37[j]
8:2	pH 4.5	–	36–44[j]
DOPE/TPE/CHEMS (1:1:1)	pH 4.5	10–35	–
	pH 7.4/3 mM Ca^{2+}	40–60	–

[a] The media also contains 100–200 mM NaCl. Dashes indicate no available data. [b] This temperature range corresponds to initial rates of NBD fluorescence increase of 0.1–1.0%/s. These are onset temperatures for the transition and should be compared with the onset temperature (T_H) for DSC measurements. [c] Epand (1985). [d] Gagné et al. (1985). [e] Ellens et al. (1986a). [f] Reiss-Husson (1967); Cullis & de Kruijff (1978b); Hardman (1982). [g] Boggs et al. (1981). [h] Mantsch et al. (1981). [i] Lai (1985); Lai et al. (1985a). [j] Bentz et al. (1985b).

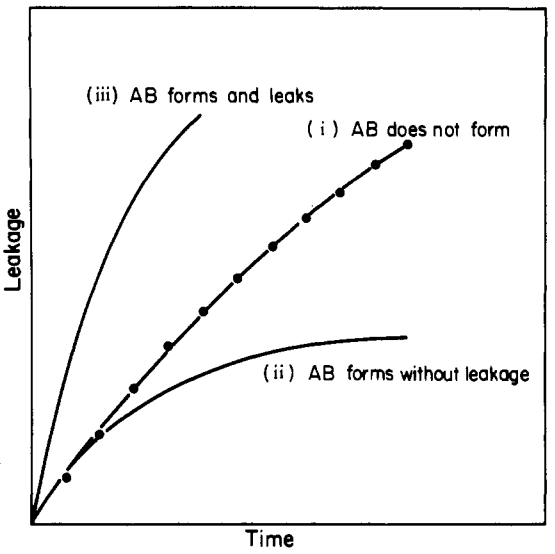

FIGURE 2: Model curves for the leakage of A-type liposomes alone [(●) dots only] and in the presence of *empty* B-type liposomes. The three solid lines represent the result of the three possible interactions between the A- and B-type liposomes. The solid line connecting the dots is the case where the B-type liposomes do not even aggregate with the A-type liposomes; hence, the leakage is identical whether the B-type liposomes are present or not. The figure is described in more detail in the text.

change much with DOPE/CHEMS (2:1) at low pH, so we used a (9:1) ratio to indicate that the T_H is decreasing with increasing mole ratios of CHEMS at pH 4.5.

Overall, these data show that these lipids are miscible and that the T_H of the binary mixtures always lies between that of the pure components. In Table I, we have collected these data and compared them with data from other sources. In Ellens et al. (1986b), other lipid types have also shown a good correlation between the NBD signal and the calorimetrically measured T_H.

Knowing the values of T_H for the various lipids, we can now study whether two liposome types, composed of different lipids, can aggregate and, if so, whether this contact induces destabilization of either or both membranes. We have chosen to monitor destabilization by the leakage of contents, using the ANTS/DPX leakage assay (Ellens et al., 1984). The quenching of ANTS fluorescence by DPX, due to collisional energy transfer, is relieved by dilution of DPX into the medium following leakage.

Monitoring destabilization at and above the H_{II} phase transition temperature by mixing aqueous contents is impossible because this mixing is abolished at and above T_H (Ellens et al., 1986a,b). In fact, the TPE/CHEMS liposomes show no H^+-induced mixing of aqueous contents at any temperature (Bentz et al., 1985b). The NBD/Rhodamine lipid mixing assay (Struck et al., 1981) is inappropriate because the increased quantum efficiency of the NBD fluorescence at and above T_H makes quantitation of lipid mixing, per se, difficult (Ellens et al., 1986a). In fact, there is no advantage here in using the lipid mixing assay over the leakage assay, once we have proven that leakage and destabilization require intermembrane contact, i.e., aggregation.

Our experimental design is idealized in Figure 2. We monitor the leakage from one type of liposomes, denoted the A type, which contain the ANTS/DPX mixture. The closed circles (●) demark these data. This experiment is then repeated in the presence of an equal concentration of B-type liposomes which are empty; i.e., they only contain buffer. In both experiments, leakage is monitored only from the A-type liposomes. The first step in the mass action kinetics of the second experiment is

$$\begin{aligned} A + A &\xrightarrow{C_{AA}} A_2 \\ A + B &\xrightarrow{C_{AB}} AB \\ B + B &\xrightarrow{C_{BB}} B_2 \end{aligned} \qquad (1)$$

where A denotes the full (ANTS/DPX) liposome, B the empty liposome, and A_2, AB, and B_2 their respective doublets. C_{AA}, C_{AB}, and C_{BB} are the forward dimerization rate constants (Bentz & Nir, 1981a,b).

In the first experiment, with only the A-type liposomes, only the first reaction is occurring ($A + A \rightarrow A_2$). In the second experiment, with both A- and B-type liposomes, there are three possible outcomes which are illustrated in Figure 2: (i) A and B do not aggregate (i.e., $C_{AB} \ll C_{AA}$). In this case, the A liposomes are unaware of the presence of the B liposomes, so the leakage curve will be identical with that of the first experiment. (ii) A and B aggregate (i.e., $C_{AB} \sim C_{AA}$), but there is no destabilization of the A liposomes. In this case, the concentration of A_2 doublets will be less than that of the first experiment, due to the formation of AB doublets. Initially, this curve (the lowest) is coincident with the first experiment, and as the AB doublets form, it falls below the leakage data of the first experiment. (iii) A and B aggregate (i.e., $C_{AB} \sim C_{AA}$), and the A-type liposome is destabilized by this contact. In this case, it is easy to understand that the leakage would be more rapid than that of the first experiment. The simplest example of this case is the mixture of A(ANTS/DPX)-type liposomes with A(buffer)-type liposomes, in which case the initial kinetics of leakage in the second experiment will be just twice as fast as that measured in the first experiment.

Figure 2 is the prototype for all of experiments, and the behavior of the leakage curve in the second experiment will tell us whether A and B can aggregate and, if so, whether the B surface can destabilize the A surface.

In Figure 3, we show that when the lipid of the A-type liposome is H_{II} competent and the lipid of the B-type liposome is L_α competent, then the AA doublet is destabilized more than the AB doublet. Conversely, an L_α-competent liposome will be destabilized much more by an H_{II}-competent liposome than by one of its own kind. It is worth noting for the Ca^{2+}-induced destabilization that only the outside monolayers of the liposomes are initially bound by Ca^{2+}. Hence, the inner monolayers are L_α competent until destabilization has allowed sufficient Ca^{2+} to enter into the liposomes (Bentz & Düzgüneş, 1985). We will examine this point below in greater detail.

Figure 3A shows the leakage from 25 μM DOPE/CHEMS (2:1) liposomes (denoted ϕ) and from these liposomes in the presence of 25 μM empty (i) DOPE/CHEMS (2:1) liposomes (denoted DOPE), (ii) TPE/CHEMS (2:1) liposomes (denoted TPE), or (iii) egg PC/CHEMS (2:1) liposomes (denoted PC). The destabilization is induced by 3 mM Ca^{2+} at 55 °C. From Table I, we know that under these conditions DOPE/CHEMS (2:1) is H_{II} competent while TPE/CHEMS (2:1) and PC/CHEMS (2:1) are L_α competent. Clearly, the DOPE/CHEMS (2:1) liposomes are destabilized best by themselves. Note that the DOPE/CHEMS (2:1) liposomes will aggregate with and destabilize on the L_α phase surfaces of TPE/CHEMS (2:1) and even PC/CHEMS (2:1).

Figure 3B shows the corresponding leakage at 55 °C in 3 mM Ca^{2+} from 25 μM TPE/CHEMS (2:1) liposomes (denoted ϕ) and in the presence of 25 μM empty (i) DOPE/CHEMS (2:1) liposomes (denoted DOPE), (ii) TPE/CHEMS

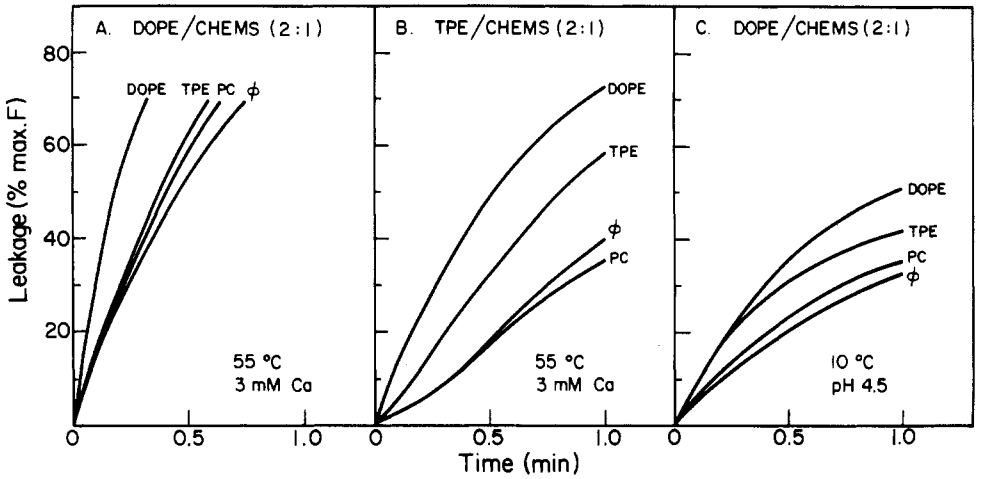

FIGURE 3: Leakage of ANTS/DPX from PE/CHEMS liposomes (LUV) alone and in the presence of empty liposomes. Panel A shows the leakage from DOPE/CHEMS (2:1) LUV (25 μM total lipid) at 55 °C induced by 3 mM Ca^{2+} (shown by the curve ϕ) and in the presence of an additional 25 μM empty DOPE/CHEMS (2:1) LUV (shown by the curve denoted DOPE), or TPE/CHEMS (2:1) LUV (shown by the curve denoted TPE), or PC/CHEMS (2:1) LUV (shown by the curve denoted PC). This notation of the data will be used throughout. Panel B shows the leakage from TPE/CHEMS (2:1) LUV (25 μM) at 55 °C induced by 3 mM Ca^{2+} (shown by the curve ϕ) and in the presence of an additional 25 μM empty DOPE/CHEMS (2:1) LUV (denoted DOPE), or TPE/CHEMS (2:1) LUV (denoted TPE), or PC/CHEMS (2:1) LUV (denoted PC). Panel C shows the leakage of DOPE/CHEMS (2:1) LUV (25 μM) at 10 °C induced by a pH drop to 4.5 in acetate buffer (shown by the curve ϕ) and in the presence of an additional 25 μM empty DOPE/CHEMS (2:1) LUV (denoted DOPE), or TPE/CHEMS (2:1) LUV (denoted TPE), or PC/CHEMS (2:1) (denoted PC).

(2:1) liposomes (denoted TPE), and (iii) PC/CHEMS (2:1) liposomes (denoted PC). Clearly, the H_{II} phase competent DOPE/CHEMS (2:1) liposomes induce much more leakage from the TPE/CHEMS (2:1) liposomes. This result can be easily understood by noting that in the area of contact where the lipid mixture is presumably ~TPE/DOPE/CHEMS (1:1:1), this mixture is at or very close to its T_H according to Figure 1. Two TPE/CHEMS (2:1) liposomes in contact give a lipid mixture in the area of contact well below its T_H. Obviously, the lipids need not be above their T_H to leak; however, being so produces substantially larger leakage rates. We find that the TPE/CHEMS (2:1) and PC/CHEMS (2:1) liposomes do aggregate together somewhat but there is no destabilization from this pair.

Figure 3C shows the leakage from DOPE/CHEMS (2:1) liposomes with the same sequence of other liposomes as in Figure 3A, except that the conditions are 10 °C and pH 4.5, using an acetate/acetic acid buffer. This case is effectively identical with that of 55 °C with 3 mM Ca^{2+} in that the DOPE/CHEMS (2:1) lipid is H_{II} competent, while the TPE/CHEMS (2:1) and PC/CHEMS (2:1) lipids are both L_α competent. Likewise, essentially the same results are found. The H_{II}-competent liposomes destabilize themselves better than L_α liposomes will.

Two points need to be made at this stage. First, this method of comparing the leakage from one set of liposomes induced by another set of liposomes is very sensitive. Differences exceeding 2% are significant and reproducible. Second, the rigorous kinetic description of this process, as alluded to by the mass action kinetics shown in eq 1, requires that the leakage process is rate limited by the liposomal aggregation. For the PE/CHEMS liposome experiments described here, this is the case. As explained in Ellens et al. (1984), when the leakage curves for different concentrations of liposomes are plotted as a function of the lipid concentration times the time and they superimpose onto the same line, then there is no significant leakage before liposomal contact, and the leakage process itself is rapid compared with the aggregation rate. For example, in Figure 3A, we see that the leakage from 25 μM DOPE/CHEMS (2:1) liposomes at 0.5 min is about 53% (see the curve labeled ϕ) and is equal to the leakage from a 50 μM sample of the same liposomes at 0.25 min (see the curve labeled DOPE).

In Figure 4, we find the very interesting result that when both A and B lipids are H_{II} competent, then the mixed doublet AB is destabilized more than either pure pair AA or AB. Figure 4A shows the leakage from DOPE/CHEMS (2:1) liposomes, and Figure 4B shows the conjugate case of the leakage from TPE/CHEMS (2:1) liposomes. The leakage is measured at 35 °C and pH 4.5, where Figure 1 and Table I show that both the DOPE/CHEMS (2:1) and TPE/CHEMS (2:1) lipids are H_{II} competent. From Figure 1 and Table I, we also know that the enhanced instability of the mixed doublet is not due to any greater propensity of the mixed lipids to form the H_{II} phase, as measured by the T_H.

Our basic conclusions also hold for liposomes composed of pure PE's, specifically DOPE, EPE, and TPE. Figure 5 shows the leakage from pure TPE liposomes at various temperatures following injection of Ca^{2+} into the pH 9.5 buffer.

In Figure 5A, the leakage from 50 μM TPE liposomes is shown (denoted ϕ) as well as the leakage from these liposomes in the presence of 50 μM empty (i) DOPE liposomes (denoted DOPE), (ii) EPE liposomes (denoted EPE), and (iii) TPE liposomes (denoted TPE). It was not possible to achieve strict aggregation rate-limiting kinetics due to the multilamellarity of these liposomes (Ellens et al., 1986a). At 5 °C, we know that DOPE is just below its T_H, while EPE and TPE are well below their respective T_H's (Table I). Likewise, the DOPE liposomes are much more destabilizing than the TPE liposomes. The leakage induced by EPE was essentially the same as the TPE curve. At 25 °C (Figure 5B), the leakage curves are qualitatively the same.

A large enhancement of the ability of the EPE liposomes to destabilize the TPE liposomes is seen at 50 °C (Figure 5C) where both EPE and the EPE/TPE (1:1) mixture are H_{II} competent (see Figure 1). Finally, at 70 °C (Figure 5D), where all of the lipids are H_{II} competent, the DOPE and EPE liposomes are somewhat better than the TPE liposomes at destabilizing the ANTS/DPX-containing TPE liposomes. It is *important* to note that this last set of data (Figure 5D) was taken with only 5 μM TPE alone (denoted ϕ) or in the presence of 5 μM empty DOPE, EPE, or TPE liposomes.

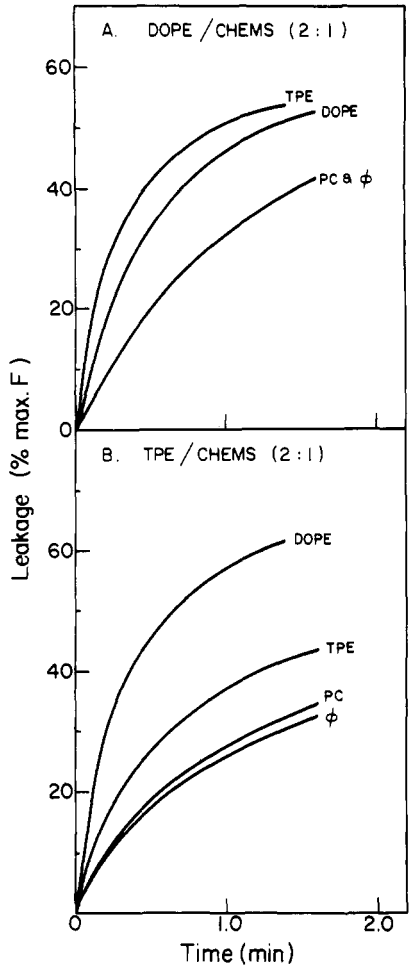

FIGURE 4: Leakage of ANTS/DPX from PE/CHEMS liposomes (LUV) alone and in the presence of empty liposomes. Panel A shows the leakage from DOPE/CHEMS (2:1) LUV (25 μM total lipid) at 35 °C induced by the pH 4.5 acetate buffer (shown by the curve denoted ϕ) and in the presence of 25 μM empty TPE/CHEMS (2:1) (LUV) (denoted TPE), or DOPE/CHEMS (2:1) LUV (denoted DOPE), or PC/CHEMS (2:1) LUV (denoted PC). Note that the leakage in the presence or absence of the PC/CHEMS liposomes is identical. Panel B shows the leakage from TPE/CHEMS (2:1) LUV (25 μM) at 35 °C induced by the pH 4.5 acetate buffer (shown by the ϕ curve) and in the presence of 25 μM empty DOPE/CHEMS (2:1) LUV (denoted DOPE), or TPE/CHEMS (2:1) LUV (denoted TPE), or PC/CHEMS (2:1) LUV (denoted PC).

Furthermore, the Ca^{2+} concentration was reduced to 5 mM. These changes were necessary since at temperatures above the T_H of the TPE, leakage was very rapid. Reducing the total lipid concentration and the Ca^{2+} concentration slowed the leakage process to a measurable rate. At 5 °C, these lipid and Ca^{2+} concentrations would not induce measurable leakage on these time scales. Note the different time scales in Figure 5C,D.

Similar results were found when the DOPE or EPE liposomes were monitored for leakage in the presence of either pure DOPE, EPE, or TPE liposomes in 20 mM Ca^{2+} at pH 9.5.

As mentioned before, in all cases of Ca^{2+}-induced destabilization, we know that initially only the outer monolayers are bound by Ca^{2+} and so the inner monolayer remains L_α competent until after destabilization and leakage are well under way. This also proves that the enhanced destabilization above the equilibrium T_H of the lipids in these cases was generated primarily, if not entirely, by the outer monolayers. However, it is difficult to get Ca^{2+} equilibrated across bilayers before the initial aggregation occurs, which is a necessary step to quantitate the effect of the asymmetry. With protons, this is not a problem.

In Figure 6, we see the effect of pH asymmetry across bilayers using a citrate/citric acid, pH 4.5, buffer, which can be compared to the acetate/acetic acid buffer in Figure 4. The difference between these two buffers is that the acetic acid can cross intact liposome bilayers very rapidly, whereas the citric acid cannot (Straubinger et al., 1983; Barbet et al., 1984). We have found for PC/CHEMS (1:1) liposomes that the pH 4.5 acetate buffer will reduce the intraliposomal pH to below 5.0 within 2 s, whereas the citrate buffer remains above pH 6.0 for at least 2 min (data not shown). Therefore, in the acetate buffer, the CHEMS on both sides of the intact liposome is instantly protonated. In citrate buffer, the CHEMS on the interior monolayer of the intact liposome is not protonated until destabilization has permitted substantial influx of the external media.

Figure 6A shows the leakage from DOPE/CHEMS (2:1) liposomes (denoted ϕ) at 35 °C and pH 4.5 in the citrate buffer. When the CHEMS is protonated, both the TPE/CHEMS (2:1) and DOPE/CHEMS (2:1) lipids are H_{II} competent. These data can be compared with the data in Figure 4A, identical except for the acetate buffer. Figure 6B shows equivalent data for the leakage from TPE/CHEMS (2:1) liposomes in the pH 4.5 citrate buffer at 35 °C. The corresponding data for leakage in acetate buffer are shown in Figure 4B. Data taken at 10 °C with loaded DOPE/

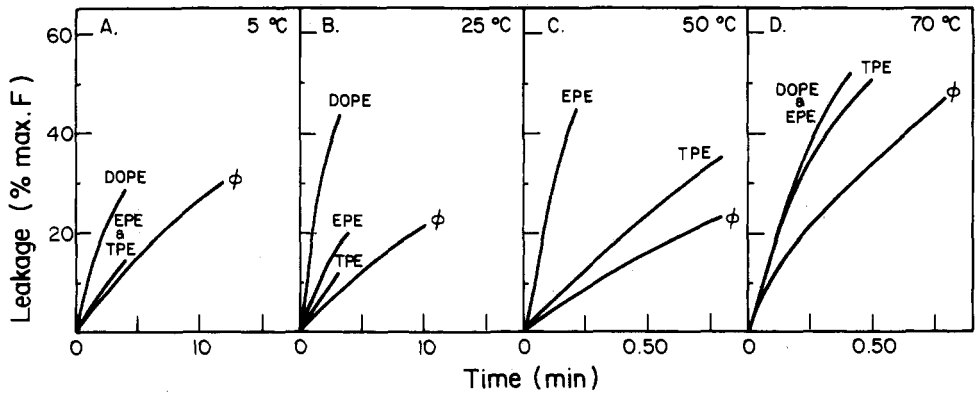

FIGURE 5: Leakage of ANTS/DPX from pure TPE liposomes (extruded MLV; see Materials and Methods) alone and in the presence of empty pure PE liposomes. All liposomes were made at pH 9.5. Panel A shows the leakage induced by 20 mM Ca^{2+} from 50 μM TPE liposomes at 5 °C (denoted by curve ϕ) and in the presence of 50 μM empty DOPE liposomes, EPE, liposomes, or TPE liposomes. Panel B shows the effect of raising the temperature to 25 °C. Panel C shows the effect of raising the temperature to 50 °C. Note the change in time scale. Panel D shows the data at 70 °C where the Ca^{2+} concentration has been reduced to 5 mM, and the lipid concentrations have been reduced to slow the leakage kinetics. Here there is 5 μM ANTS/DPX-containing TPE liposomes and 5 μM empty DOPE, EPE, or TPE liposomes.

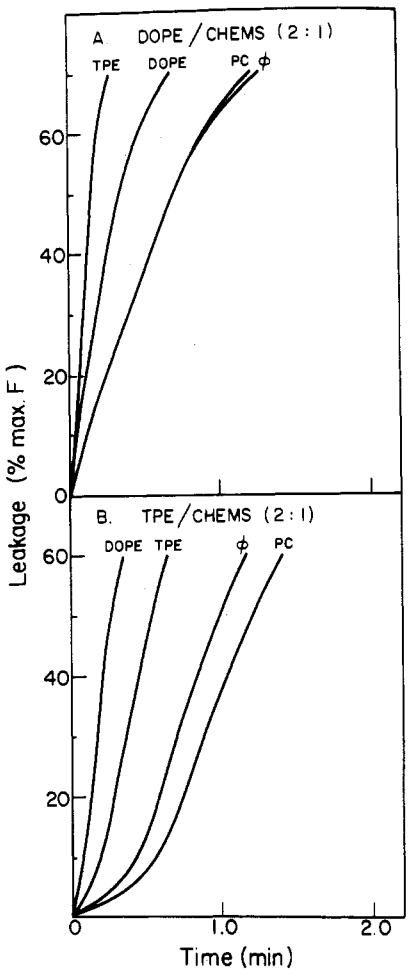

FIGURE 6: Leakage of ANTS/DPX from PE/CHEMS liposomes (LUV) at pH 4.5 in the citrate buffer. Panel A shows the leakage from DOPE/CHEMS (2:1) LUV (25 μM total lipid) at 35 °C induced by the pH 4.5 citrate buffer (shown by the curve denoted ϕ) and in the presence of 25 μM empty TPE/CHEMS (2:1) LUV (denoted TPE), or DOPE/CHEMS (2:1) LUV (denoted DOPE), or PC/CHEMS (2:1) LUV (denoted PC). Panel B shows the leakage from the TPE/CHEMS (2:1) LUV (25 μM total lipid) at 35 °C induced by the pH 4.5 citrate buffer (shown by the curve denoted ϕ) and in the presence of 25 μM empty DOPE/CHEMS (2:1) LUV (denoted DOPE), or TPE/CHEMS (2:1) LUV (denoted TPE), or PC/CHEMS (2:1) LUV (denoted PC).

CHEMS (2:1) liposomes in the citrate buffer were qualitatively similar to those of Figure 3C, except that the empty TPE/CHEMS (2:1) liposomes did not significantly destabilize the DOPE/CHEMS (2:1) liposomes after aggregation (data not shown). Therefore, our main conclusion is the same: the mixed doublet AB is more unstable, and the protonated outer surface of the PE/CHEMS liposomes above T_H is sufficient to enhance destabilization. However, the shapes of the leakage curves are quite different. The leakage in the citrate buffer is eventually faster.

In Figure 7, we show the leakage from DOPE/CHEMS (2:1) (25 μM) (Figure 7A–C) or from TPE/CHEMS (2:1) (25 μM) (Figure 7D–F) at pH 4.5 using either the acetate (solid line) or the citrate (dashed line) buffer. In Figure 7D, only, there is an additional 25 μM empty DOPE/CHEMS (2:1) liposomes, because there was no measurable leakage from the TPE/CHEMS liposomes alone under these conditions. Now, it is very clear that the leakage in the citrate buffer is eventually faster, even in Figure 7D. Furthermore, we can see that the acetate buffer promotes more leakage initially: having both sides of the bilayer H_{II} competent leads to a greater initial destabilization. Leakage in the citrate buffer

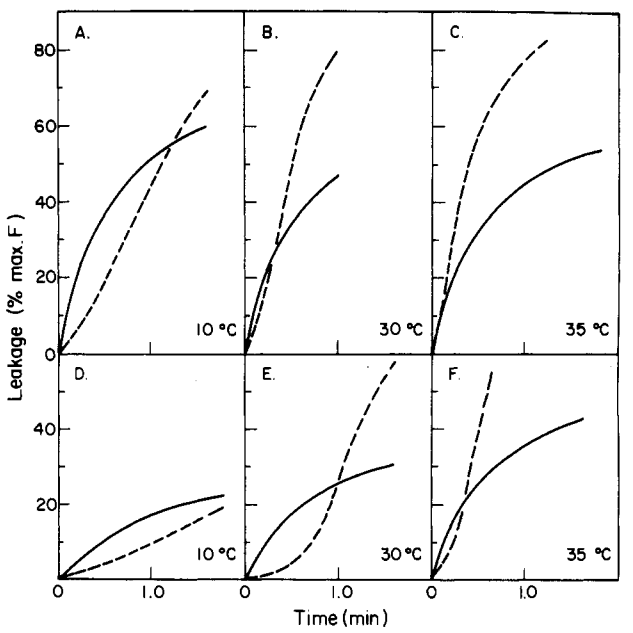

FIGURE 7: Leakage of ANTS/DPX from PE/CHEMS liposomes (LUV) at pH 4.5 using either an acetate/acetic acid buffer (solid line) or a citrate/citric acid buffer (dashed line). Panels A–C show the leakage from 25 μM DOPE/CHEMS (2:1) LUV at 10, 30, and 35 °C, respectively. Panels D–F show the leakage from 25 μM TPE/CHEMS (2:1) LUV at 10, 30, and 35 °C, respectively. In panel D only, there is an additional 25 μM empty DOPE/CHEMS (2:1), because the TPE/CHEMS liposomes show no leakage at 10 °C on this time scale.

shows a lag phase. For DOPE/CHEMS at 35 °C, the compensating reaction responsible for the greater extent of leakage in the citrate buffer occurs too rapidly to permit observance of any lag phase.

The mechanism of this second reaction is not known, but we suspect that bilayer flip-flop of the protonated CHEMS is involved. To test this, we measured the leakage from the pure TPE liposomes (10 μM) at pH 4.5 in the acetate and citrate buffers. Both at 60 °C, above T_H, and at 40 °C, below T_H, the leakage did not depend upon which buffer was used (data not shown). Without the CHEMS, there was no greater initial destabilization in the acetate buffer nor a greater eventual destabilization in the citrate buffer. It is worth noting here that in both cases, PE/CHEMS (2:1) at pH 7.4 and TPE at pH 9.5, the number of protons which must penetrate the membrane in order to lower the internal pH to, e.g., 5.0 is essentially the same and is almost completely determined by the number of lipid molecules which must be protonated in each case. A 2000-Å diameter sphere at pH 5.0 contains only 25 free hydronium ions. The surface of this sphere would require about 10^5 CHEMS molecules (2:1 PE/CHEMS) or 2×10^5 TPE molecules to cover it. The recently reported pK_a of EPE is 9.6 (Tsui et al., 1986). Therefore, at pH 9.5, about half of those TPE molecules would be protonated already. Since the difference in leakage kinetics between the acetate and citrate buffers only occurs in PE/CHEMS liposomes and not in PE liposomes, it must be due to the presence of CHEMS in the bilayer.

Discussion

In Bentz et al. (1985b) and Ellens et al. (1986a), we showed that the contact-mediated leakage from PE-containing liposomes was vastly enhanced when the temperature was at or above the T_H, i.e., when the lipids could undergo the L_α–H_{II} phase transition. The transition evidently begins at the level of two apposed liposomes. Here we show that only one of the

liposomes needs to be H_{II} phase competent in order to promote the enhanced destabilization and leakage. Biological membranes are often asymmetric, and it cannot be expected that both membranes will be H_{II} competent at the same time, i.e., in the same environment. Therefore, this result was required to sustain the speculation that the L_α–H_{II} phase transition is one mechanism of biological fusion (Cullis & de Kruijff, 1979; Verkleij, 1984; Siegel, 1984; Gruner et al., 1985).

We have also shown that liposome fusion, i.e., mixing of aqueous contents, is abolished at and above T_H; i.e., only leakage and lipid mixing occurs (Bentz et al., 1985b; Ellens et al., 1986a,b). Such an outcome is not surprising, once it was known that the liposomes start turning into H_{II} phase tubes as soon as they touch another (identical) liposome. This does not imply that the L_α–H_{II} transition is irrelevant to biological fusion, which requires concomitant mixing of aqueous contents and membrane components.

First, we now have evidence that those polymorphic phospholipids which can sustain the isotropic intermediate state between the lamellar L_α and the hexagonal H_{II} phases can also sustain a proper liposome fusion event (Ellens et al., 1986b). For these phospholipids, this isotropic state is very hysteretic and shows up as isotropic ^{31}P NMR resonances, lipidic particles, or cubic X-ray diffraction patterns (Cullis & de Kruijff, 1978a; Verkleij et al., 1979, 1982; Hui et al., 1981b, 1983; Sen et al., 1981; Hui & Boni, 1982; Borovjagin et al., 1982; Tilcock et al., 1982; Williams et al., 1982; Gagné et al., 1985). It has been proposed for some lipids that this isotropic state represents a metastable intermediate stage between the L_α and H_{II} phases (Larsson et al., 1980; Hui et al., 1983; Siegel, 1986a–c, 1987). In Ellens et al. (1986b), we proposed that the molecular mechanism which underlies the very long lifetimes of this metastable state is also responsible for the liposome fusion event. Thus, the L_α–H_{II} phase transition is relevant to fusion for those lipid mixtures which can begin, but not rapidly complete, the transition to the H_{II} phase. It should be noted that other lipids and surfactants do form a true cubic phase, which also gives isotropic ^{31}P NMR resonances and cubic X-ray diffraction patterns (Rilfors et al., 1984).

Second, this phase transition is a powerful mechanism for membrane destabilization. With two apposed membranes, a fluctuation leading to a local lipid composition which is H_{II} competent in *either* apposed monolayer would be sufficient for destabilization, as we have shown here. To complete the fusion event, the interior monolayers must participate, and, most importantly, the destabilization must relax back to bilayers; otherwise, there will be no mixing of aqueous contents.

Another important, if somewhat unexpected, result was that when the DOPE/CHEMS or TPE/CHEMS liposomes were H_{II} competent, they could often (if not always) destabilize upon contact with a PC/CHEMS surface. While contact is required to induce the destabilization of the PE-containing liposomes, that contact can be with some very stable surfaces. It seems possible that any surface to which the H_{II}-competent liposomes could bind is a potentially destabilizing surface. This result may explain the leakage found by Ho and Huang (1985) from DOPE/*N*-[[(dinitrophenyl)amino]caproyl]-PE liposomes following their adhesion to anti-Dnp-IgG-coated glass surfaces. Lateral phase segregation of the Dnp-Cap-PE toward the glass surface would leave domains too rich in DOPE to remain stable after contact with other surfaces, perhaps including the glass itself.

Mixed Membrane Destabilization. Another conclusion reached here pertains directly to the molecular mechanism of the L_α–H_{II} phase transition. We find that when both liposome types are H_{II} competent, the mixed doublet AB shows greater destabilization than either of the pure pairs AA or BB.

We cannot yet explain this result; however, we can describe the meaning of the kinetics involved in this observation. To do so, we must begin with the general initial mass action reaction for these liposomes which accounts for aggregation reversibility and the rate of destabilization of dimers:

$$\begin{aligned} A + A &\underset{D_{AA}}{\overset{C_{AA}}{\rightleftharpoons}} A_2 \xrightarrow{f_{AA}} F(A_2) \\ A + B &\underset{D_{AB}}{\overset{C_{AB}}{\rightleftharpoons}} AB \xrightarrow{f_{AB}} F(AB) \\ B + B &\underset{D_{BB}}{\overset{C_{BB}}{\rightleftharpoons}} B_2 \xrightarrow{f_{BB}} F(B_2) \end{aligned} \tag{2}$$

where A and B denote the monomers of the A-type and B-type liposomes, A_2, AB, and B_2 are the aggregated dimers of the liposomes, and $F(A_2)$, F(AB), and $F(B_2)$ denote the destabilized doublets, i.e., where the membranes have mixed and leakage of contents has occurred. f_{AA}, f_{AB}, and f_{BB} denote the destabilization rate constants for this process. D_{AA}, D_{AB}, and D_{BB} are the dimer dissociation rate constants for the reversion of the aggregated dimers back to the isolated monomers. Higher order reactions will occur, but as we have shown for the aggregation and fusion/leakage of a homogeneous population of liposomes, the initial reactions can be used to qualitatively describe the real situation (Bentz et al., 1983a). Nir et al. (1986) have used these reactions, with higher orders, to analyze virus particle–liposome fusion kinetics.

We believe that the explanation of the greater destabilization of the mixed doublets lies in the values of the aggregation kinetic rate constants per se. We have chosen the lipid concentrations to provide aggregation rate-limiting kinetics (as far as possible) in order to simplify the interpretation of the data. Under the simplest conditions, this implies that the leakage kinetics directly reflect the values of C_{AA}, C_{AB}, and C_{BB}. This would imply that above T_H, the aggregation of A and B is more rapid than that of either of the pure pairs. We will speculate below on why this might be the case; however, first it must be noted that aggregation rate-limiting kinetics for leakage or liposome fusion need *not* always imply that aggregation reversibility and the destabilization rate constant, e.g., D_{AB} and f_{AB}, are irrelevant to the observed results. It only implies that the observed leakage kinetics depend upon the product of $C_{AB}X_0t/(1 + D_{AB}/f_{AB})$, where X_0 is the liposome concentration and t is the time, together with the corresponding terms for the pure pairs AA and BB (Bentz et al., 1983a; unpublished data). *Only if* the dimers destabilize much more rapidly than they can dissociate, i.e., $f_{AB} \gg D_{AB}$, do the overall aggregation rate-limiting kinetics directly, and uniquely, reflect the aggregation rate constant, C_{AB}.

The aggregation rate constant C_{AB} is rigorously defined by the integral of a function of the free energy of interaction between the two liposomes over their distance of separation up to the point of the energy minimum which holds them together. Once in this energy minimum, either they can drift apart, where the value of D_{AB} becomes involved, or they establish the molecular contact required to destabilize the apposed bilayers, where the value of f_{AB} becomes involved (Nir & Bentz, 1978; Bentz & Nir, 1981a,b; Bentz et al., 1983a). This free energy depends upon many factors, e.g., electrostatic repulsion, van der Waals attraction, and short-range forces (Bentz et al., 1985a), which include solvation forces (Rand, 1981; Marra & Israelachvili, 1985). Since all evidence on the L_α–H_{II} transition supports the model that interbilayer contact

is required to start the transition (Gruner et al., 1982; Siegel, 1984, 1986a,b; Ellens et al., 1984, 1986a,b; Bentz et al., 1985b), it is difficult to imagine that the bilayers are altered sufficiently before contact to radically change the electrostatics and van der Waals forces. However, it is known that the H_{II} phase is more dehydrated (per molecule) than the L_α phase (Cullis & de Kruijff, 1978a; Siegel, 1984; Verkleij, 1984; Gruner et al., 1985) and that the lamellar water spacing decreases as the temperature approaches T_H, using X-ray diffraction of multilamellar dispersions (Hui et al., 1983). Therefore, it could be that above T_H the membranes of the asymmetric (AB) liposomes do not engage as much bound water as either of the pure pairs, which would reduce the solvation force and selectively enhance C_{AB}.

On the other hand, if the aggregation is reversible below T_H (i.e., $D_{AB} \sim f_{AB}$), then the data could imply that above T_H, aggregation becomes more irreversible for the mixed dimer. D_{AB} will decrease if the energy minimum holding the dimer together becomes deeper and/or the energy barrier which inhibits aggregation becomes higher (Bentz & Nir, 1981a,b; Shoup & Szabo, 1982). This could happen in two distinct ways. First, if the solvation force is diminished, as described above, then the energy minimum holding the dimer together could become deeper. A second mechanism for reducing aggregation reversibility lies precisely within the mechanism of the L_α–H_{II} phase transition. Roughly speaking, the L_α–H_{II} phase transition can be described by two steps. First, there is the formation of some intermembrane attachments (IMI) between the H_{II}–competent bilayers, and later, the H_{II} tubes form either from aggregation of the IMI, from line defects emanating from the IMI, or possibly from some other mechanism (Verkleij et al., 1979; Hui et al., 1981a, 1983; Miller, 1980; Sen et al., 1981; Rand et al., 1981; Gruner et al., 1982, 1985; Verkleij, 1984; Siegel, 1984, 1986a,b, 1987a,b; Gagné et al., 1985; Caffrey, 1985; Ellens et al., 1985b; Rilfors et al., 1986). For the liposomes in close apposition, the formation of just a few IMI connecting the liposomes would decrease their ability to dissociate. This could be the mechanism of the enhanced destabilization of the mixed dimer in that the mixed IMI may form more readily than those composed of the pure components. In this case, only a few IMI need to exist, and a strong reduction of the calorimetric T_H for the lipid mixture is not necessarily expected, since it only monitors the temperature of facile formation of H_{II} tubes.[2]

Role of the Inner Monolayer. Our final conclusion concerns the extent to which the inner monolayer of the liposome affects the overall contact-induced destabilization. The data in Figure 4, using DOPE/CHEMS (2:1) and TPE/CHEMS (2:1) liposomes, showed that when both inside and outside monolayers were H_{II} competent, i.e., in acetate buffer where all of the CHEMS is protonated, the destabilization is initially faster than when only the outer monolayers are H_{II} competent, i.e., in citrate buffer where the inside monolayers initially remain at pH 7.4. This result makes sense in light of the L_α–H_{II} phase transition since the inside monolayers must participate in the transition in order to promote leakage.

The unexpected finding was that *eventually* the leakage from the liposomes in the citrate buffer was faster. Something more than a simple equilibration of pH has taken place, due to influx of medium into the liposomes following destabilization (Bentz & Düzgüneş, 1985). Since the destabilization and leakage from pure TPE liposomes did not depend upon which buffer was used, we believe that the explanation is a redistribution of molecules following protonation of the CHEMS.

Once CHEMS is protonated, it becomes very similar to cholesterol (Lai et al., 1985a,b), which is well-known for its ability to flip-flop across membranes (Lange et al., 1981; Yeagle, 1985). In acetate buffer, all of the CHEMS is protonated, and the inside–outside distribution of CHEMS remains constant since the flip-flop rate is the same in both directions. In citrate buffer, initially only the outside CHEMS is protonated and, hence, capable of transfer to the inside monolayer, possibly, just in the area of interliposomal contact and destabilization. Once there, like all of the CHEMS initially residing in the inside monolayers, they are deprotonated in the pH 7.4 buffer and, as charged molecules, severely inhibited from motion back across the bilayer. This net flow of CHEMS to the inner monolayers of the liposomes would increase the PE content of the outer monolayers. We have shown before that while adding CHEMS to PE at pH 4.5 lowers the T_H (Lai et al., 1985a; Bentz et al., 1985b), more and faster leakage occurs from liposomes with less CHEMS, provided the temperature remains above T_H (Ellens et al., 1984; Bentz et al., 1985b). Therefore, these outer monolayers in the citrate buffer may have a higher PE content than the liposomes in acetate, and this may promote the eventual enhanced leakage in the citrate buffer.

There is a separate issue concerning asymmetry across the membrane which we have not yet addressed. The binding of a cation to a lipid will change, to some extent, the equilibrium area per head group. When that binding occurs only on the outside monolayer of a liposome, then some surface tension gradient between the inner and outer monolayers will be established, which should affect the stability of the membranes (Papahadjopoulos & Ohki, 1969). This situation occurs with the Ca^{2+}-induced fusion of PS liposomes, as well as the Ca^{2+}-induced destabilization of the PE/CHEMS liposomes or the H^+-induced destabilization of these PE/CHEMS liposomes when the citric acid buffer is used. No asymmetry occurs when the acetic acid buffer is used. The magnitude of the contribution of this surface tension asymmetry to the destabilization of the apposed membranes is not known. However, for the PE/CHEMS liposomes, the experiments show that the initial destabilization is greater in the acetate buffer, where there is no asymmetry, than in the citrate buffer, where there is an initial asymmetry. With the pure TPE liposomes, there was no difference between the two buffers. It is unlikely that the surface tension gradient stabilizes the membranes. What is likely is that compared with the desta-

[2] We do not believe that the greater destabilization of the mixed doublet F(AB) is due to a greater leakage per contact. If this were the case, then it would be necessary that, above T_H, the pure doublets F(AA) and F(BB) do not completely leak; i.e., the complete leakage observed occurs due to the formation of higher order aggregates. Incomplete leakage for liposomes above T_H is not impossible, but all of our data suggest rapid, complete leakage under these conditions (Bentz et al., 1985b; Ellens et al., 1986a,b). Greater leakage of the F(AB) doublet would also occur if the destabilization rate constant f_{AB} was larger than f_{AA} and f_{BB}. As noted in the text, under the aggregation rate limited kinetics used in our experiments, the values of the destabilization rate constants become observable *only if* the aggregation is also quite reversible. In any event, we expect that the magnitudes of these destabilization rate constants depend strongly, if not solely, upon the rate of propagation of the L_α–H_{II} phase transition. The study of these rates has only just begun (Caffrey, 1985; Siegel, 1986a, 1987a,b), but it is certain that they will depend upon the excess temperature above T_H, as well as upon other specific properties of the lipids. Simply put, we would predict that if the T_H of the mixed lipid AB were lower than that of either of the pure components, then f_{AB} should be greater than either f_{AA} or f_{BB}. However, as shown here and previously by van Dijck et al. (1976) and Tilcock and Cullis (1982), the T_H of the miscible mixture of two H_{II}–competent lipids lies roughly between the T_H's of the pure components. Thus, if f_{AB} is greater than f_{AA} and f_{BB}, the explanation is not easily apprehended.

bilization promoted when both inner and outer monolayers are H_{II} phase competent, the effect of the surface tension gradient is rather insignificant.

Conclusion

The data presented here, together with the kinetic and physical arguments presented under Discussion, have produced three major conclusions. The polymorphism displayed by lipids in the L_α–H_{II} phase transition is a powerful agent of membrane destabilization between apposed bilayers, even if only the exterior monolayer of one of the bilayers is H_{II} competent. In fact, the ability of one monolayer to initiate the L_α–H_{II} phase transition with an apposed membrane would appear to be necessary for this molecular mechanism to be relevant to biological fusion. Second, when the lipids of two dissimilar liposomes, A and B, are H_{II} competent, then the destabilization of the mixed doublet F(AB) is greater than that of either of the pure pairs F(AA) or F(BB). This is true even though the T_H of the mixed lipid system lies between that of the two pure components, i.e., A and B. We believe this reflects differences in liposome aggregation rates and reversibility, rather than in the membrane destabilization rate constants. This may imply that the hydration force between membranes is sensitive to this asymmetry above T_H. Third, we found that while the low-pH contact-induced destabilization of the PE/CHEMS liposomes is initially more rapid when both sides of each bilayer are H_{II} competent, the collapse of the liposomes in a citrate buffer, which initially produces only an outer monolayer of H_{II}-competent lipid, was eventually faster. The simplest explanation is that when CHEMS is protonated, and quite similar to cholesterol, the flip-flop of the CHEMS to the inner monolayer leads to a higher PE content on the exterior monolayers, which is known to further promote destabilization.

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