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Articles

Destabilization of Phosphatidylethanolamine Liposomes at the Hexagonal Phase Transition Temperature†

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ABSTRACT: We have examined whether there is a relationship between the lamellar–hexagonal phase transition temperature, T_H, and the initial kinetics of H^+- and Ca^{2+}-induced destabilization of phosphatidylethanolamine (PE) liposomes. The liposomes were composed of dioleoylphosphatidylethanolamine, egg phosphatidylethanolamine (EPE), or phosphatidylethanolamine prepared from egg phosphatidylcholine by transesterification (TPE). These lipids have well-spaced lamellar–hexagonal phase transition temperatures (~12, ~45, and ~57 °C) in a temperature range that allows us to measure the initial kinetics of bilayer destabilization, both below and above T_H. The liposomes were prepared at pH 9.5. The T_H of EPE and TPE was measured by using differential scanning calorimetry, and it was found that the T_H was essentially the same at low pH or at high pH in the presence of 20 mM Ca^{2+}. At temperatures well below T_H, either at pH 4.5 or at pH 9.5 in the presence of Ca^{2+}, the liposomes aggregate, leak, and undergo lipid mixing and mixing of contents. We show that liposome/liposome contact is involved in the destabilization of the PE liposomes. The temperature dependence of leakage, lipid mixing, and mixing of contents shows that there is a massive enhancement in the rate of leakage when the temperature approaches the T_H of the particular PE and that lipid mixing appears to be enhanced. However, the fusion (mixing of aqueous contents) is diminished or even abolished at temperatures above T_H. At and above the T_H, a new mechanism of liposome destabilization arises, evidently dependent upon the ability of the PE molecules to adapt new morphological structures at these temperatures. We propose that this destabilization demarks the first step in the pathway to the eventual formation of the H_{II} phase. Thus, the polymorphism accessible to PE is a powerful agent for membrane destabilization, but additional factors are required for fusion.

One of the most intensively examined phospholipids is phosphatidylethanolamine (PE).[1] The reason for this is that PE can form nonbilayer structures under physiological conditions, i.e., lipidic particles and the tubular H_{II} phase [see Verkleij (1984) for a review]. The uniqueness of PE in this regard has led to the speculation that it may be integrally involved in the control of cellular fusion since any fusion event necessarily requires the transitory existence of nonbilayer structures (Cullis & de Kruijff, 1979; Verkleij, 1984).

The property of PE which makes this speculation amenable to experimental analysis is that each PE has a phase transition temperature range (T_H) which demarks the morphological change from bilayer to H_{II} phase in the direction of increasing temperature (Verkleij, 1984). The acyl chain composition of the PE determines T_H. Mixing the particular PE with other

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[1] Abbreviations: PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; EPE, egg phosphatidylethanolamine; TPE, phosphatidylethanolamine prepared from egg phosphatidylcholine by transesterification; PS, phosphatidylserine; PC, phosphatidylcholine; 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); LUV, large unilamellar vesicle(s); SUV, small unilamellar vesicle(s); T_H, temperature range demarking the lamellar–hexagonal H_{II} phase transition; T_c, temperature range demarking the lamellar gel–liquid-crystalline phase transition; EDTA, ethylenediaminetetraacetic acid; MLV, multilamellar vesicle(s); DSC, differential scanning calorimetry.

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lipids, e.g., cholesterol (Cullis & de Kruijff, 1978; Tilcock et al., 1982), cholesteryl hemisuccinate (Lai et al., 1985), or PS (Tilcock & Cullis, 1981), or varying the pH (Seddon et al., 1983) or ionic strength (Harlos & Eibl, 1981) will alter the T_H, sometimes even abolishing the transition. Provided that the variations are not too drastic, in most cases there is an identifiable T_H below which the lipids are in the bilayer or lamellar configuration and above which the PE and perhaps the other lipids are in the H_{II} phase (Tilcock et al., 1982). Therefore, if the polymorphism accessible to PE is relevant to the destabilization of two apposed liposomes (whether this leads to mixing of contents, lipid mixing, or leakage), then this destabilization should be markedly enhanced as the temperature approaches T_H.

It is important to recall that the measurement of T_H for PE-containing lipid systems is done for the equilibrium structures accessible to the lipids, be they multilamellar, multitubular, or arrays of lipidic particles (Verkleij et al., 1979; Cullis et al., 1980; Hui et al., 1981; Rand et al., 1981; Sen et al., 1981). The T_H is an equilibrium property of the lipids. On the other hand, the fusion of two liposomes composed of the same lipids is a nonequilibrium event, which demarks the first step of the pathway to the new equilibrium state. There does not have to be a connection between the equilibrium T_H and the temperature dependence of the initial kinetics of liposome destabilization. For example, the ionotropic gel–liquid-crystalline phase transition temperature of PS in the presence of Ba^{2+} and Sr^{2+} is unrelated to the initial kinetics of PS liposome fusion induced by these cations (Düzgünes et al., 1984; Bentz et al., 1985a). In addition, it is known that PS/PE liposomes will fuse in Ca^{2+} and Mg^{2+} under conditions where there is no H_{II} phase formation even at equilibrium (Düzgünes et al., 1981b, 1984). With cardiolipin/PC liposomes in Ca^{2+}, the observance of lipidic particles by electron microscopy occurs only after long incubations, well after the initial fusion events (Bearer et al., 1982; Wilschut et al., 1985). What remains is the following essential question: At temperatures where the H_{II} phase *is* accessible to the lipids, is there an enhancement of bilayer destabilization between two apposed liposomes, and, if so, does it lead to fusion, i.e., mixing of aqueous contents? This is the issue upon which a substantial body of literature rests.

In spite of the importance of this issue, this work and our previous study (Bentz et al., 1985b) are the first to experimentally address it. It was known that liposomes composed of different ratios of TPE (a PE prepared from the transesterification of egg PC) and CHEMS (cholesteryl hemisuccinate) undergo a lamellar–hexagonal phase transition at pH 4.5 where CHEMS is protonated (Lai et al., 1985). In Bentz et al. (1985b), it was shown that when TPE/CHEMS LUV were injected into pH 4.5 buffers there was a massive enhancement of leakage and lipid mixing at and above the temperature range of the T_H. However, there was *no* mixing of aqueous contents with these liposomes at any temperature. Hence, these liposomes do respond to the equilibrium T_H via an enhanced contact-mediated lysis.

In this paper, we have examined the relationship between T_H and the initial kinetics of H^+- and Ca^{2+}-induced destabilization of liposomes using pure DOPE (dioleoylphosphatidylethanolamine), EPE (egg yolk phosphatidylethanolamine), and TPE transesterified from egg yolk PC. The PE liposomes were prepared at pH 9.5. Incubation of the liposomes at pH 4.5 or at pH 9.5 in the presence of Ca^{2+} leads to aggregation, leakage, lipid mixing, and fusion. We show that interbilayer contact is required for the destabilization of the PE liposomes. We have measured the temperature dependence of this destabilization and show that the rate of leakage is enhanced when the temperature approaches the T_H of the particular PE but that the fusion (mixing of aqueous contents) is abolished at temperatures above T_H. Since cellular fusion entails the destabilization and mixing of the apposed membranes, as well as the coalescence of the aqueous contents, it is now clear that the polymorphism accessible to PE-containing membranes above T_H can enhance the kinetics of membrane destabilization but other membrane components must mitigate this action to ensure the coalescence of the encapsulated contents. A preliminary report of these results has been published (Ellens et al., 1985a).

Materials and Methods

Dioleoylphosphatidylethanolamine (DOPE), egg phosphatidylethanolamine (EPE), phosphatidylethanolamine prepared by transesterification from egg phosphatidylcholine (TPE), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) prepared from TPE, and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine prepared from dioleoylphosphatidylethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL). 1-Aminonaphthalene-3,6,8-trisulfonic acid (disodium salt) (ANTS) and *p*-xylylenebis(pyridinium bromide) (DPX) were from Molecular Probes Inc. (Junction City, OR). ANTS gave a single spot when tested by thin-layer chromatography with chloroform/methanol/acetic acid/water (100:50:14:16) and chloroform/methanol/ammonia/water (115:45:2:6) and was considered chromatographically pure.

The TPE, EPE, and DOPE liposomes were prepared according to Szoka & 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 ANTS, ANTS/DPX, or DPX in glycine buffer, pH 9.5, or just glycine buffer, pH 9.5; see below). 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 mixture was vortexed vigorously until the lipid was completely dispersed. The liposome suspension was then maintained under high vacuum (water aspirator) for 45 min to remove residual ether.

For the ANTS/DPX leakage and fusion assay (Ellens et al., 1984, 1985b), 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. For the lipid mixing experiments, the liposomes contained glycine buffer (100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA, pH 9.5). In all cases, the encapsulated solutions were isoosmotic to the buffers used for the column chromatography and in the leakage, lipid mixing, and fusion experiments. The liposomes were extruded through polycarbonate membranes with 0.1-μm pores (Olson et al., 1979). The liposomes were separated from unencapsulated material on Sephadex G-75 (Pharmacia) using 100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA (pH 9.5) as elution buffer. Liposomal lipid concentrations were determined by phosphate analysis (Bartlett, 1959).

The size distribution of the liposomes was measured by using dynamic light scattering (Coulter Model N4, Coulter Electronics, Inc., Hialeah, FL). The TPE liposomes for leakage, fusion, and lipid mixing were 235 ± 83 nm (*Z*-average diameter), the EPE liposomes were 243 ± 85 nm, and the DOPE liposomes were 359 ± 118 nm. The encapsulated volume of the ANTS/DPX-containing liposomes was 3.1, 4.6, and 6.1

μL/μmol of lipid for the TPE, EPE, and DOPE liposomes, respectively, and for the ANTS-containing liposomes 3.4, 4.6, and 15.2 μL/μmol of lipid for the TPE, EPE, and DOPE liposomes, respectively. It is clear from these data that, although PE liposomes can be made and extruded at pH 9.5, the resulting liposomes are oligomeric and larger than, for example, pure PS liposomes made by the same procedure (Bentz et al., 1985a). Even at pH 9.5, where PE is negatively charged, the extrusion of PE yields larger liposomes. Also the size distribution of the PE liposomes varied between preparations. The encapsulated contents of liposomes stored at 4 °C showed no measurable leakage for at least several days.

We determined that there was no significant binding of ANTS to the exterior of the liposomes by incubating "empty" DOPE, EPE, and TPE liposomes with the ANTS and ANTS/DPX mixtures used for liposome preparation for 60 min at room temperature. The medium was separated from the liposomes on Sephadex G-75, and the remaining fluorescence associated with the liposomes was ≤1% of the total fluorescence that the liposomes would have encapsulated. The binding of DPX to the PE liposomes cannot be directly assayed. The fact that it is possible to make PE liposomes in at least 90 mM DPX indicates that *if* there is any binding of DPX to PE, this binding is very weak [see also Ellens et al. (1985b)].

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. 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., 1985b). The fluorescence scale is calibrated with the fluorescence of a 1:1 mixture of ANTS and DPX liposomes in glycine buffer (100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA, pH 9.5) taken as 100% fluorescence (0% fusion). The 0% fluorescence level was set with the appropriate buffer, since the residual fluorescence of liposomes containing 12.5 mM ANTS and 45 mM DPX is close to zero. Excitation was at 360 nm and emission >530 nm. On the figures, fusion (percent maximum 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. It should be noted that ANTS fluorescence is not sensitive to pH between 9.5 and 4.5 and also that the quenching efficiency of DPX is not affected by pH in this region (results not shown).

Lipid mixing was monitored as described by Struck et al. (1981). Liposomes composed of TPE, EPE, or DOPE containing 1 mol % each of the fluorescent lipid analogue NBD-PE and Rh-PE were mixed with respectively TPE, EPE, or DOPE liposomes devoid of fluorescent lipids. The ratio of fluorescent to nonfluorescent liposomes was 1:9. Lipid mixing is registered as an increase in fluorescence from the NBD probe which is due to decreased energy transfer between NBD-PE and Rh-PE as the two probes are diluted. The 100% fluorescence level was set with respectively TPE, EPE, or DOPE liposomes containing 0.1 mol % each of NBD-PE and Rh-PE at the same total lipid concentration as used in the lipid mixing experiment. This was done because Triton X-100 severely quenches NBD fluorescence (Tanaka & Schroit, 1983). Sodium cholate has the same effect. The 100% fluorescence level in the H^+-induced lipid mixing experiments was set immediately after injection of the 0.1% NBD/Rh liposomes, at $t = 0$, into the pH 4.5 buffer. In the Ca^{2+}-induced lipid mixing experiments, the 100% fluorescence level was set immediately after injection of the $CaCl_2$, at $t = 0$, into the 0.1% NBD/Rh liposome suspension. Excitation was at 450 nm, and emission was measured at 530 nm.

Ca^{2+}- and H^+-induced fusion (mixing of aqueous contents) and lipid mixing 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 glycine buffer (100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA, pH 9.5). 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). In *all* cases, the buffers were made isoosmotic to the encapsulated contents of the liposomes by 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.

For differential scanning calorimetry (DSC), MLV lipid dispersions were prepared in 10 mM glycine, 100 mM NaCl, and 0.1 mM EDTA (pH 9.5) at a lipid concentration of 20 μmol/mL. The liposomes were dialyzed for 3 h against three changes of 100 mL of the pH 4.5 acetate/acetic acid buffer (50 mM acetate/acetic acid and 66 mM NaCl) or the Ca^{2+}-containing pH 9.5 buffer (10 mM glycine, 100 mM NaCl, 0.1 mM EDTA, and 20 mM $CaCl_2$). Samples for DSC (200 μL) were concentrated by centrifugation in an Eppendorf centrifuge (12800g, 3 min), and the pellet was dispersed in ~50 μL of the buffer used for dialysis. Approximately 17 μL of this suspension was sealed in an aluminum sample pan, and the DSC scans were performed on a Perkin-Elmer DSC-2 calorimeter operating at a sensitivity of 1 mcal/s and at a scanning rate of 5 °C/min. Continuous heating and cooling runs were performed on each sample. The heating curves showed peaks at 8–11 °C higher than those found for the subsequent cooling curves. Similar results have been seen by Cullis & de Kruijff (1978) and Yager & Chang (1983) and have been predicted from theory (Siegel, 1984). The EPE liposomes at pH 4.5 were also scanned at a rate as low as 1.25 °C/min, and this gave the same results as the 5 °C/min scan. The beginning and end points of the lamellar–H_{II} transition on the heating curves are taken as the hexagonal phase transition temperature range.

Results

PE liposomes can be made at high pH (>9.0) where PE is negatively charged (Stollery & Vail, 1977). DMPE liposomes made at pH 9.0 and incubated at low pH (<7.0), where PE is protonated and zwitterionic, will aggregate (Kolber & Haynes, 1979) and show lipid mixing (Pryor et al., 1985). In order to fully comprehend the destabilization of PE liposomes, we followed the fate of both the encapsulated molecules and the lipids, measuring the H^+- or Ca^{2+}-induced leakage, fusion, and lipid mixing of the liposomes over a temperature range covering the transition to the hexagonal phase. We chose DOPE, EPE, and TPE because they have well-spaced hexagonal phase transition temperatures (~8–18, ~30–45, and ~50–63 °C, respectively).

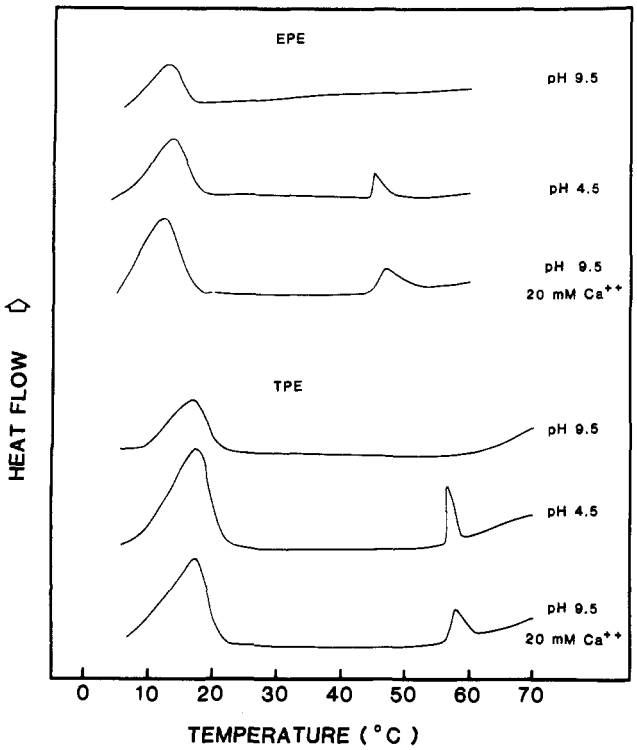

FIGURE 1: Differential scanning calorimetry of EPE and TPE at pH 9.5, 4.5, and 9.5 in the presence of Ca^{2+}. The MLV lipid suspensions were prepared at pH 9.5 in 10 mM glycine, 100 mM NaCl, and 0.1 mM EDTA at a lipid concentration of 20 μmol/mL; 0.6 mL of the suspension was dialyzed against pH 4.5 buffer (50 mM acetate/acetic acid and 66 mM NaCl) or pH 9.5 buffer with $CaCl_2$ (10 mM glycine, 100 mM NaCl, 0.1 mM EDTA, and 20 mM $CaCl_2$). Continuous heating and cooling scans were performed at a scanning rate of 5 °C/min.

The T_H of DOPE is well established (Cullis & de Kruijff, 1978; Gagné et al., 1985); however, the T_H of PE's from natural sources may vary between batches, probably due to differences in acyl chain compositions (Lai et al., 1985). We therefore measured the T_H of EPE and TPE at pH 4.5 and at pH 9.5 in the presence of 20 mM Ca^{2+} using differential scanning calorimetry (Figure 1). At pH 4.5, EPE showed a transition to the hexagonal phase between 43 and 47 °C (beginning and end point of the transition on the heating curve) and TPE between 56 and 59 °C. At pH 9.5 in the presence of 20 mM Ca^{2+}, EPE showed this transition between 45 and 50 °C and TPE between 56 and 62 °C. As has been shown before (Cullis & de Kruijff, 1978), there is no hexagonal transition visible in DSC when PE is dispersed at pH 9.5. It is interesting that both H^+ and Ca^{2+} will induce the phase transition at almost the same temperature.

Temperature Dependence of H^+- and Ca^{2+}-Induced Leakage of Liposomes Composed of DOPE, EPE, and TPE. The kinetics of H^+-induced leakage from DOPE, EPE, and TPE liposomes was measured at various temperatures at pH 4.5. Figure 2 shows the leakage curves of the DOPE liposomes between 5 and 30 °C, as indicated in the figure. The rate of leakage from these liposomes shows a marked increase around T_H. The EPE and TPE liposomes showed very similar behavior at their respective T_H ranges. This is indicated in Figure 3, which shows the temperature dependence of the initial rates of leakage for the three sets of liposomes. For convenience, the lamellar–hexagonal phase transition temperature range is shown in the figure by the horizontal arrows, marked T_H. For each PE, the initial rate of leakage increases dramatically when the temperature approaches T_H. Similar results were obtained with the Ca^{2+}-induced destabilization of the PE liposomes, albeit at somewhat higher temperatures. Figure 4 shows the temperature dependence of the initial rates of leakage when the liposomes were incubated with 20 mM Ca^{2+} at pH 9.5. It is important to note that PE liposomes at pH

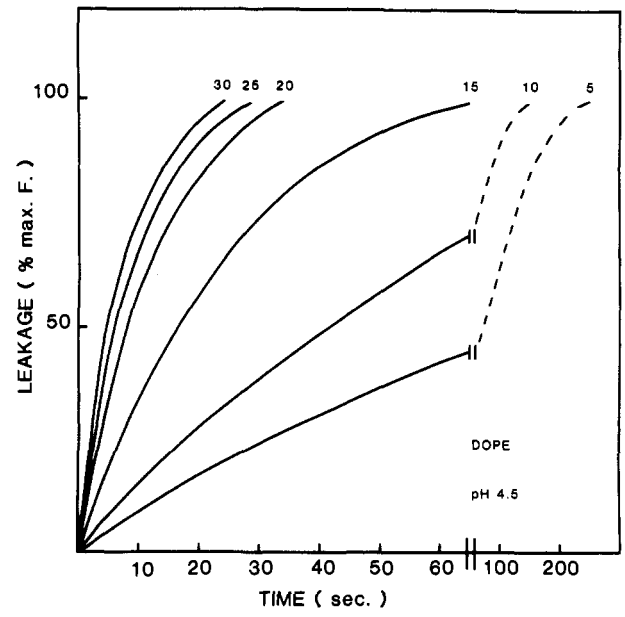

FIGURE 2: H^+-induced leakage of DOPE liposomes at various temperatures. Leakage is measured by the release of the pH-insensitive fluorophore ANTS from the liposomes into the medium. ANTS fluorescence in the liposomes is quenched by collisional energy transfer to DPX. At t = 0, DOPE liposomes, 40 μM total lipid, containing both ANTS and DPX were injected into 1 mL of the pH 4.5 buffer (50 mM acetate/acetic acid and 66 mM NaCl). The incubations were done at 5, 10, 15, 20, 25, and 30 °C. Leakage is measured as described under Materials and Methods.

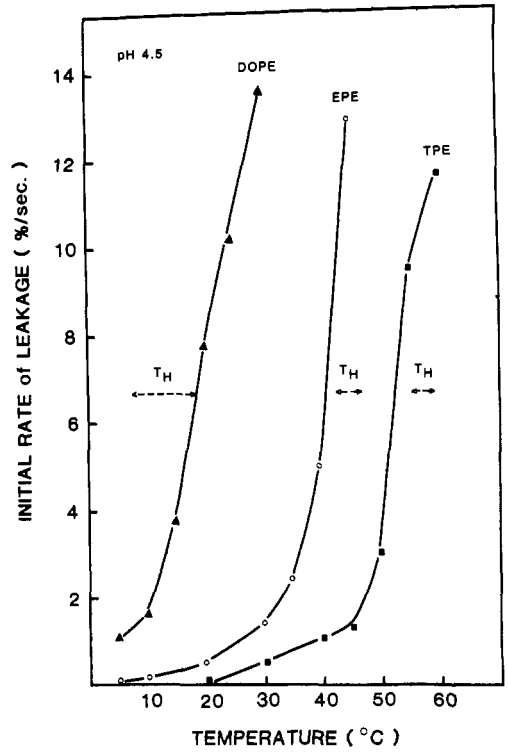

FIGURE 3: Initial rates of H^+-induced leakage of DOPE, EPE, and TPE liposomes shown as a function of temperature. DOPE (40 μM), TPE (40 μM), and EPE (20 μM) liposomes containing both ANTS and DPX were injected into 1 mL of the pH 4.5 buffer (50 mM acetate/acetic acid and 66 mM NaCl) at various temperatures. The initial rates are just the initial slopes of the leakage curves (in units of percent per second) as shown in Figure 2 for the DOPE liposomes.

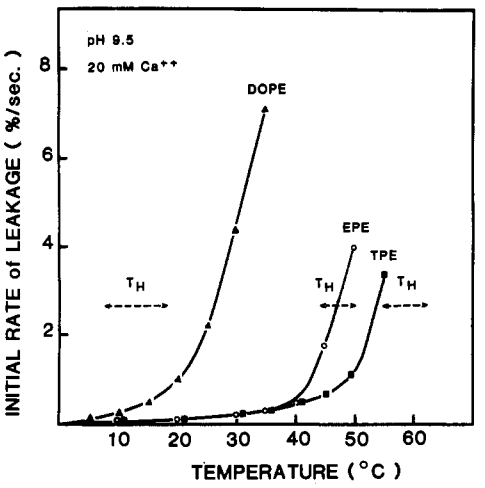

FIGURE 4: Initial rates of Ca^{2+}-induced leakage of DOPE, EPE, and TPE liposomes shown as a function of temperature. DOPE (40 μM), TPE (40 μM), and EPE (20 μM) liposomes containing both ANTS and DPX were suspended into 1 mL of the pH 9.5 buffer (10 mM glycine, 100 mM NaCl, and 0.1 mM EDTA). The incubations were done at various temperatures. At t = 0, 20 mM $CaCl_2$ was injected, and leakage was measured as described under Materials and Methods. The initial rates are just the initial slopes of the leakage in units of percent per second.

9.5 in the absence of Ca^{2+} do not aggregate and show essentially no leakage. For example, the TPE liposomes at 60 °C leak less than 1%/min.

Thus, there is a very clear response of the initial rates of H^+- and Ca^{2+}-induced leakage of DOPE, EPE, and TPE liposomes to T_H. On the other hand, it is also quite clear from Figures 2–4 that leakage does occur below T_H, where the H_{II} phase can play no role. At 5 °C, the DOPE liposomes show complete release with H^+ in 3–4 min and 75% release with Ca^{2+} in about 15 min. At 20 °C, the EPE liposomes show about 70% release in 5–6 min with either H^+ or Ca^{2+}, and at 10 °C, the TPE liposomes release about 55% of their contents in a 5-min incubation with H^+ or Ca^{2+}. In these cases, the equilibrium state is that of extensive lamellar sheets. Since DMPE shows no H_{II} phase transition under the experimental condition of Pryor et al. (1985), it is evident that the liposome destabilization monitored in that study arose from the same mechanism. There are clearly two general mechanisms by which PE liposomes are destabilized, each with their own temperature dependence. The interaction of these two mechanisms is at least partially responsible for the relatively broad temperature dependence of the enhanced initial rates of liposome destabilization near T_H. This will be discussed in more detail below.

Change in Quantum Efficiency of 0.1% NBD/Rh Liposomes at or above the Lamellar to Hexagonal Phase Transition Temperature. The next step is showing the temperature dependence of lipid mixing. However, before doing so, it is necessary to elucidate a very interesting "problem" with the NBD/Rh assay which somewhat obscures the observation of enhanced lipid mixing at the T_H, but which also appears to provide a new and very sensitive fluorometric method for determining the onset of the lamellar to H_{II} phase transition.

For the lipid mixing assay, the 100% fluorescence level used to indicate complete lipid mixing is set with liposomes containing 0.1 mol % each of NBD-PE and Rh-PE (0.1% NBD/Rh liposomes) at the same total lipid concentration as used in the lipid mixing experiment. The fluorescence of the 0.1% NBD/Rh liposomes does not change when the liposomes aggregate and undergo lipid mixing below T_H. However, when the temperature is at or above the T_H, there is a time-dependent increase in the fluorescence of these liposomes when they are incubated at low pH or with Ca^{2+} (Figure 5). The temperature at which the fluorescence of the 0.1% NBD/Rh liposomes starts to increase correlates remarkably well with the hexagonal phase transition temperatures measured by NMR for DOPE (Cullis & de Kruijff, 1978) and by DSC for EPE and TPE (Figure 1).

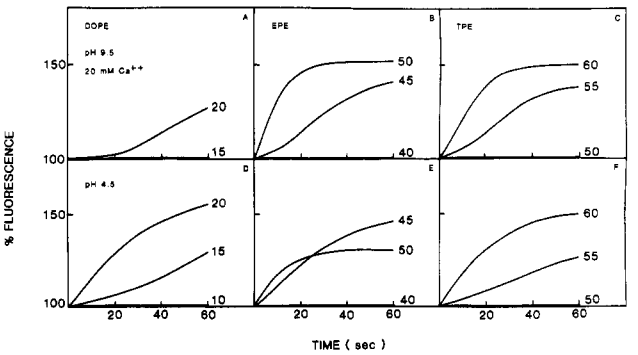

FIGURE 5: Effect of temperature on the fluorescence of liposomes containing 0.1 mol % NBD-PE and Rh-PE. (A–C) 40 μM 0.1 mol % NBD/Rh DOPE liposomes (panel A), 20 μM 0.1 mol % NBD/Rh EPE liposomes (panel B), or 40 μM 0.1 mol % NBD/Rh TPE liposomes (panel C) were incubated in pH 9.5 glycine buffer (10 mM glycine, 100 mM NaCl, and 0.1 mM EDTA) at various temperatures as indicated in degrees centigrade next to the fluorescence curves. At t = 0, 20 mM $CaCl_2$ was injected into the liposome suspensions. The fluorescence scale was calibrated, as in a lipid mixing experiment, with the fluorescence of a 1:9 mixture of 1.0 mol % NBD/Rh liposomes and unlabeled liposomes at t = 0 as 0% fluorescence and with the fluorescence of the 0.1 mol % NBD/Rh liposomes at t = 0 as 100% fluorescence. (D–F) At t = 0, 40 μM 0.1 mol % NBD/Rh DOPE liposomes (panel D), 20 μM 0.1 mol % NBD/Rh EPE liposomes (panel E), or 40 μM 0.1 mol % NBD/Rh TPE liposomes (panel F) were injected into 1 mL of a pH 4.5 buffer (50 mM acetate/acetic acid and 66 mM NaCl) at various temperatures as indicated in the figure in degrees centigrade next to the fluorescence curves. The fluorescence scale was calibrated, as in a lipid mixing experiment, with the fluorescence of a 1:9 mixture of 1 mol % NBD/Rh liposomes and unlabeled liposomes at t = 0 as 0% fluorescence and with the fluorescence of the 0.1 mol % NBD/Rh liposomes at t = 0 as 100% fluorescence.

Since there are no unlabeled liposomes, the NBD and rhodamine probes are already maximally separated, and even if the rhodamine were removed entirely, as by phase separation, the NBD fluorescence would increase by less than 5% (Struck et al., 1981). Thus, the increase in fluorescence represents an increase in the quantum efficiency of NBD-PE (rather than a decrease in energy transfer), due to a change in the local environment of the probes, e.g., refractive index or dielectric constant, as the lipids go from lamellar to nonbilayer structures. We confirmed this by monitoring the temperature-dependent change in fluorescence of PE liposomes containing 0.1 mol % of NBD-PE and no Rh-PE (0.1% NBD liposomes). The initial rates of the increase in fluorescence of the 0.1% NBD liposomes and the 0.1% NBD/Rh liposomes are shown as a function of temperature in Figure 6. It is clear that the NBD fluorescence is enhanced at and above T_H for both types of liposomes. Similar observations have been made for many other H_{II} phase competent lipids (K. Hong, P. A. Baldwin, T. M. Allen, and D. Papahadjopoulos, unpublished results). Below we will show that bilayer contact mediates this transition.

Temperature Dependence of H^+- and Ca^{2+}-Induced Lipid Mixing of Liposomes Composed of TPE, EPE, and DOPE. Figure 7 shows the kinetics of H^+-induced lipid mixing of TPE liposomes at pH 4.5, at the temperatures indicated. The EPE and DOPE liposomes showed similar lipid mixing kinetics, but, of course, at different temperatures. The fluorescence of the

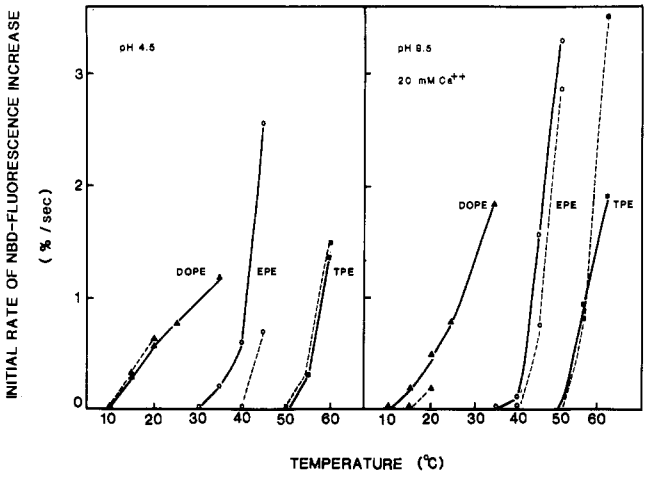

FIGURE 6: Initial rates of the H^+- and Ca^{2+}-induced increase in fluorescence of 0.1 mol % NBD/Rh liposomes and 0.1 mol % NBD liposomes at temperatures around T_H. DOPE, EPE, and TPE liposomes containing either 0.1 mol % NBD/Rh (dashed lines) or 0.1 mol % NBD (solid lines) were incubated at various temperatures in a pH 4.5 buffer (left panel) or in a pH 9.5 buffer containing 20 mM Ca^{2+} (right panel). The fluorescence scale was calibrated with the fluorescence of the 0.1 mol % NBD/Rh liposomes or the 0.1 mol % NBD liposomes at $t = 0$ as 100% fluorescence and buffer as 0% fluorescence. The initial rates are just the initial slopes of the increase in fluorescence in units of percent per second. For the 0.1 mol % NBD/Rh liposomes, the initial rates were taken from the experiment shown in Figure 5. The 0.1 mol % NBD liposomes were incubated at a lipid concentration of approximately 20 μM.

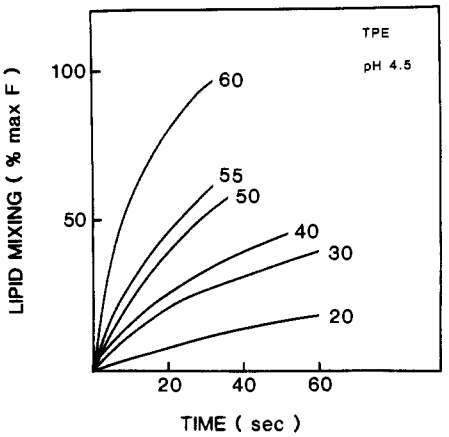

FIGURE 7: H^+-induced lipid mixing of TPE liposomes at various temperatures. At $t = 0$, a 40 μM 1:9 mixture of 1.0 mol % NBD/Rh TPE liposomes and unlabeled TPE liposomes was injected into 1 mL of a pH 4.5 buffer (50 mM acetate/acetic acid and 66 mM NaCl). The incubations were done at various temperatures, as indicated in degrees centigrade next to the curves. Lipid mixing is measured by the increase in NBD fluorescence, due to a decreased energy transfer to rhodamine, caused by dilution of the fluorescent probes into the pool of unlabeled lipid. The fluorescence scale was calibrated with the fluorescence of 40 μM 0.1 mol % NBD/Rh TPE liposomes at $t = 0$ as 100% lipid mixing and with the fluorescence of a 40 μM 1:9 mixture of 1.0 mol % NBD/Rh TPE and unlabeled TPE liposomes at $t = 0$ as 0% lipid mixing.

1.0% NBD/Rh liposomes (in a 50 μM aliquot of the 1:9 mixture of labeled and unlabeled liposomes) at $t = 0$, immediately after injection of the liposomes, was taken as 0% lipid mixing. The 100% fluorescence level was set at the temperature of the incubation, with 50 μM 0.1% NBD/Rh liposomes at pH 4.5. Because of the change in fluorescence quantum efficiency at and above the T_H, in these cases the 100% fluorescence level was set, somewhat arbitrarily, with the fluorescence of the 0.1% NBD/Rh liposomes at $t = 0$ immediately after injection of the liposomes, i.e., the 100% levels

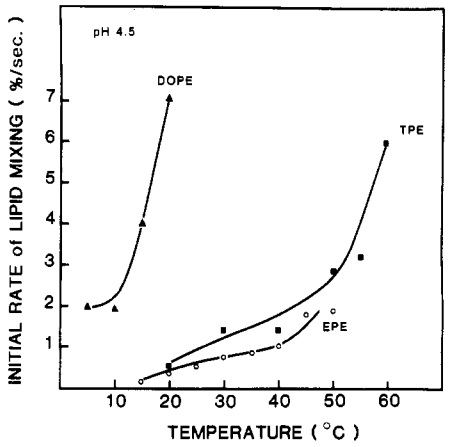

FIGURE 8: Initial rates of H^+-induced lipid mixing of DOPE, EPE, and TPE liposomes as a function of temperature. DOPE (40 μM), EPE (20 μM), or TPE (40 μM) liposomes were injected into 1 mL of the pH 4.5 buffer at various temperatures (for details, see legend of Figure 6). The initial rates are just the initial slopes of the lipid mixing curves (in units of percent per second) as shown in Figure 7 for the TPE liposomes.

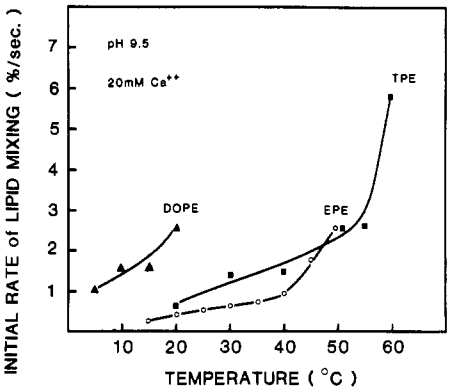

FIGURE 9: Initial rates of Ca^{2+}-induced lipid mixing of DOPE, EPE, and TPE liposomes as a function of temperature. DOPE (40 μM), EPE (20 μM), or TPE (40 μM) liposomes were incubated in pH 9.5 glycine buffer (10 mM glycine, 100 mM NaCl, and 0.1 mM EDTA) at various temperatures. At $t = 0$, 20 mM $CaCl_2$ was injected, and lipid mixing was measured as described under Materials and Methods. The fluorescence scale was calibrated with the fluorescence of a 1:9 mixture of 1 mol % NBD/Rh liposomes and unlabeled liposomes at $t = 0$ as 0% lipid mixing and with the fluorescence of the 0.1 mol % NBD/Rh liposomes at $t = 0$ as 100% lipid mixing.

shown in Figure 5. Another way of setting the 100% fluorescence level would be to let the 0.1% NBD/Rh liposomes go to their equilibrium structure at pH 4.5. However, since at or above T_H the lipids precipitate, such a calibration is not feasible.

The purpose of the calibration is to correlate a certain increase in fluorescence with a certain amount of lipid mixing. Because of the change in fluorescence of the 0.1% NBD/Rh liposomes at T_H, the same increase in fluorescence below T_H and above T_H does not indicate the same amount of lipid mixing. There is no simple, rigorous solution for this problem. The calibration we use here will overestimate the extent of lipid mixing above T_H. On the other hand, a 100% setting based upon the equilibrium intensity, even if it could be taken, would underestimate the lipid mixing kinetics.

Figures 8 and 9 show the temperature dependence of the initial rates of H^+- and Ca^{2+}-induced lipid mixing. The NBD/Rh lipid mixing assay shows an enhancement at the T_H of the lipids with both Ca^{2+} and H^+, but the increase in the fluorescence quantum efficiency at these temperatures makes it difficult to quantitate the direct effect upon the lipid mixing

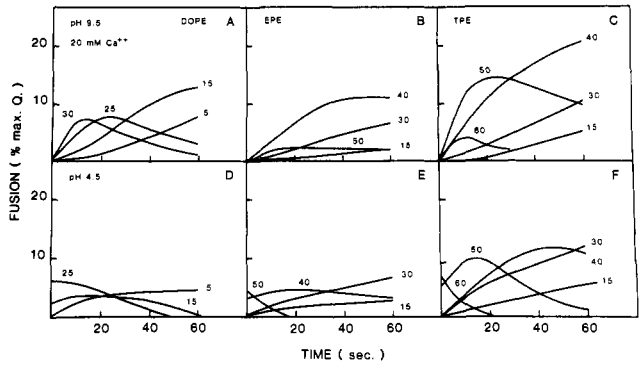

FIGURE 10: H^+- and Ca^{2+}-induced mixing of aqueous contents of DOPE, EPE, and TPE liposomes at various temperatures. A 40 μM 1:1 mixture of ANTS- and DPX-containing DOPE liposomes (panels A and D), a 20 μM 1:1 mixture of ANTS- and DPX-containing EPE liposomes (panels B and E), and a 40 μM 1:1 mixture of ANTS- and DPX-containing TPE liposomes (panels C and F) were incubated at pH 9.5 with 20 mM Ca^{2+} (panels A–C) or at pH 4.5 (panels D–F) at various temperatures as indicated in degrees centigrade next to the fusion curves. Note that the fusion curves are given in the units of percent maximum quenching, i.e., 100 minus the measured percent maximum fluorescence signal.

kinetics. It is important to realize that the initial rates of leakage as shown in Figures 3 and 4 cannot be compared directly to the initial rates of lipid mixing as shown in Figures 8 and 9, because the assays are not calibrated in the same way. For example, if all the liposomes dimerized, complete leakage would lead to a 100% increase in fluorescence, but complete lipid mixing, as doublets, would only lead maximally to a 30% increase in fluorescence (Struck et al., 1981; Allen & Düzgüneş, 1985).

Temperature Dependence of H^+- and Ca^{2+}-Induced Aqueous Contents Mixing of Liposomes Composed of DOPE, EPE, and TPE. The leakage experiments very clearly show that there is a sudden and dramatic increase in the rate of bilayer destabilization upon aggregation of the PE liposomes at temperatures around T_H and the lipid mixing experiments are in agreement with this observation. Bilayer destabilization can lead to leakage and/or mixing of contents (fusion). In Figure 10, we show the temperature dependence of the H^+- and Ca^{2+}-induced mixing of contents of the DOPE, EPE, and TPE liposomes using the ANTS/DPX fusion assay described under Materials and Methods (Ellens et al., 1985b). First of all, it is very interesting to note that H^+ and Ca^{2+} can actually induce the fusion of PE liposomes below T_H. Surprisingly, the extents of fusion do not increase very much with temperature and in fact decrease at temperatures above T_H. Thus, it can be seen in Figure 10 that the extent of Ca^{2+}-induced fusion of TPE liposomes is decreased quite significantly at 60 °C, of EPE liposomes at 50 °C, and of the DOPE liposomes at 25 °C.

The H^+-induced fusion shows a very similar temperature dependence behavior. The fusion signal essentially disappears at 60, 50, and 25 °C for TPE, EPE, and DOPE liposomes, respectively. At these temperatures, at time zero, there is a certain amount of quenching, ~7% for TPE, ~4% for EPE, and ~6% for DOPE liposomes, which only decreases in time. This initial quenching is due to an injection "offset", since during the time of injection and mixing of the liposomes in the cuvette (~2 s) a transitory high concentration of liposomes initially at room temperature is exposed to the low-pH and high-temperature buffer. Under the conditions of the experiment, i.e., when the liposomes in the cuvette are completely mixed and temperature equilibrated, there is no significant additional fusion occurring, and the decrease in the quenching signal is due to leakage from the fused liposomes. In the Ca^{2+}-induced fusion experiments, we do not see this injection offset, since the liposomes are preincubated in the pH 9.5 buffer and the Ca^{2+} is injected into the liposome suspension.

Necessity of Interbilayer Contact for Leakage. A critical element for understanding the mechanism of liposome destabilization is discerning to what extent interbilayer contact is necessary. In the simplest form, this question can be stated as how does the leakage of the liposome after aggregation and contact with another bilayer, i.e., initially following the reaction

$$V_1 + V_1 \underset{D_{11}}{\overset{C_{11}}{\rightleftharpoons}} V_2 \xrightarrow{f_{11}} F_2 \qquad (1)$$

compare with the leakage of an isolated liposome, i.e.

$$V_1 \xrightarrow{l_1} F_1 \qquad (2)$$

A rigorous kinetic analysis of these reactions shows that if only reaction 1 is occurring, then the graph of percent leakage vs. X_0t (where X_0 is the total lipid concentration and t is time) will converge to a single curve at sufficiently low lipid concentrations (Bentz et al., 1983; Ellens et al., 1984). If only reaction 2 is occurring, the percent leakage will be independent of the lipid concentration. In Ellens et al. (1984), we showed that H^+-induced leakage from PE/CHEMS liposomes required liposome contact, for systems both below and above their T_H.[2] This was done by showing that leakage depended only upon the product of X_0t once the lipid concentration was sufficiently low.

If both reaction 1 and reaction 2 are occurring, the leakage will depend upon the lipid concentration, but not solely upon the product X_0t. What would be predicted is that at infinitely low lipid concentrations the leakage would be completely first order, following reaction 2, since reaction 1 would have a vanishingly small rate of occurrence. As the lipid concentration is increased, there will be a regime wherein the leakage depends essentially only upon X_0t, as reaction 1 becomes dominating. At higher lipid concentrations, the leakage kinetics will become first order again as the reaction $V_2 \rightarrow F_2$ becomes rate limiting (Bentz et al., 1983a; Ellens et al., 1984).

Figure 11 shows the graphs of leakage and enhanced NBD fluorescence (using just the 0.1% NBD-containing liposomes) for the TPE liposomes, plotted as a function of X_0t. The leakage curves (Figure 11A,B) clearly show this mixed kinetic behavior, with essentially cographing from 40 to 80 μM lipid and a first-order dependence at 4 μM lipid. The enhanced quantum efficiency of NBD fluorescence shows the mixed kinetic dependence for 20 mM Ca^{2+}, but at pH 4.5, it appears that only reaction 1, i.e., interliposomal contact, is kinetically relevant. At this point, it is worthwhile mentioning that the parameter which determines whether one will see the mixed dependence, as in Figure 11D, or a strict dependence on X_0t, as in Figure 11C, is the ratio $l_1:f_{11}$ for the particular system (J. Bentz, unpublished results). For Figure 11C, it may be expected that lower lipid concentrations would eventually permit the emergence of the first-order collapse process. Since the PE liposomes are oligomeric, it is very likely that the first-order process is simply due to contact between the bilayers within each liposome.

[2] Ellens et al. (1984) showed that at pH 4.5, liposomal contact was required to induce leakage from liposomes composed of DOPE/CHEMS (1:1) and TPE/CHEMS (7:3 and 8:2). Subsequent work showed that for the DOPE/CHEMS (1:1) liposomes the experimental temperature of 25 °C was well above the T_H of the lipid (Lai et al., 1985). The experimental temperature was within the T_H for TPE/CHEMS (7:3) and below the T_H for TPE/CHEMS (8:2) (Bentz et al., 1985b).

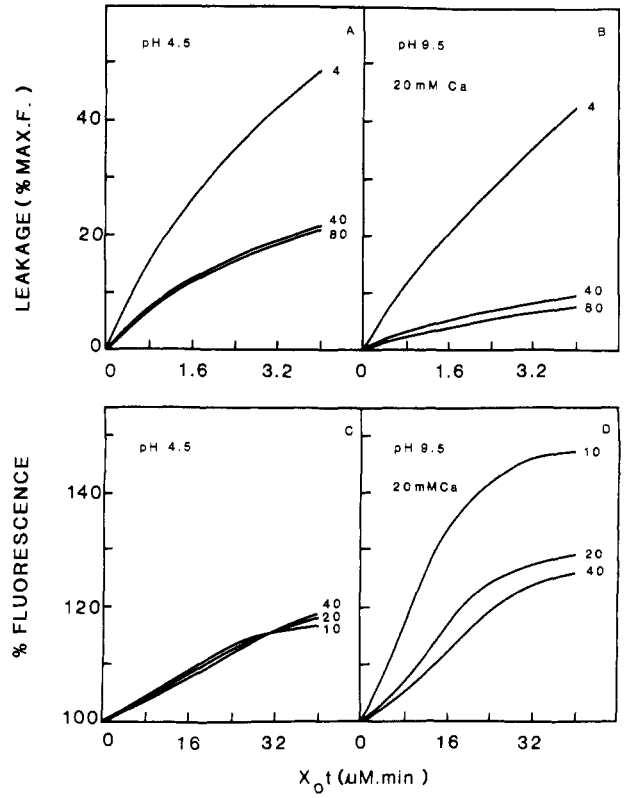

FIGURE 11: Role of bilayer contact in the induction of liposome destabilization. Panels A and B show the H^+- and Ca^{2+}-induced leakage from TPE liposomes at various lipid concentrations (indicated in micromolar next to the curves) as a function of X_0t. Panels C and D show the H^+- and Ca^{2+}-induced enhancement of NBD fluorescence in TPE liposomes (containing 0.1 mol % NBD/Rh) at various lipid concentrations (indicated in micromolar next to the curves) as a function of X_0t. Note the different scales on the abscissas. The leakage experiments were done at 50 °C, and the increase in NBD fluorescence was measured at 55 °C. For calibration of the fluorescence scales, see the legends to Figures 2 and 5.

Discussion

The study of the mechanism by which liposomes composed of a particular lipid or lipid mixture fuse has usually begun with the equilibrium properties of the lipid. For PS, it was noted that the gel–liquid-crystalline phase transition temperature, T_c, was increased in the presence of divalent cations (Papahadjopoulos et al., 1977; Portis et al., 1979). This led to the hypothesis that the divalent cation induced fusion of PS liposomes was driven by an isothermal phase transition of the apposed sections of the liposomes from liquid-crystalline to the gel phase (Papahadjopoulos et al., 1977). Recently, it has been shown that PS liposomes induced to fuse by Ba^{2+} and Sr^{2+} will do so at high temperatures where the acyl chains are in the liquid-crystalline state throughout the process, from PS LUV to the equilibrium structures (Düzgüneş et al., 1984; Bentz et al., 1985a). However, while this ionotropic shift in the T_c is not necessary to produce fusion, it is also known that PS liposomes fuse little or very slowly at low temperatures where the acyl chains are initially in the gel state (Wilschut et al., 1985). Thus, there is this correlation between the T_c of the liposomes initially in the Na^+ buffer and their fusogenicity. The opposite correlation occurs for liposomes composed of saturated PC, e.g., DMPC and DPPC, where it has been shown that the average liposome size increases only at temperatures below the T_c of these liposomes (Suurkuusk et al., 1976; Schullery et al., 1980; Wong et al., 1982). In this case, the additional requirement is that the liposomes begin as SUV, as their subsequent growth in size ceases when they reach 700–900 Å in diameter.

There is extensive literature on the competence of PE-containing (as well as cardiolipin-containing) lipid mixtures to adopt the H_{II} phase or to produce lipidic particles [see Cullis & de Kruijff (1969), Siegel (1984), and Verkleij (1984) for reviews]. The abundance of PE in biological membranes and its propensity to form nonbilayer structures underline a number of models which examine how the various morphologies accessible to the PE-containing lipid systems could facilitate the fusion of biological membranes (Cullis & de Kruijff, 1978; Verkleij et al., 1979; Hui et al., 1981; Miller, 1980; Rand et al., 1981; Siegel, 1984; Verkleij, 1984; Gruner et al., 1985).

Only recently, however, has the effect of the T_H on the initial destabilization kinetics of liposomes been examined (Bentz et al., 1985b). This study used TPE/cholesteryl hemisuccinate (CHEMS) liposomes induced to aggregate and destabilize by H^+. Briefly, the results were that at pH 4.5, where the CHEMS is protonated and is essentially cholesterol, the liposomes showed a vast enhancement of leakage of contents at temperatures within and above the T_H. Since it was known that these liposomes required interbilayer contact, i.e., between apposed liposomes, before they would leak (Ellens et al., 1984), it was clear that the apposed liposomal bilayers collapsed into pre-H_{II} phase structures (Siegel, 1984) which only led to the release of encapsulated contents. It was also shown that the mixing of bilayer components, using the lipid mixing assay of Struck et al. (1981), as was done here, showed a similar enhancement at about 10 °C below the T_H. This indicated that, while the bilayer mixing also responded to the T_H, it occurred at a somewhat lower temperature. Finally, and perhaps most importantly, there was no mixing of aqueous contents between the liposomes at any temperature. Thus, here was a lipid system for which the formation of nonbilayer structures between the apposed liposomes led straight to lysis and not fusion of aqueous contents. This study showed the obvious necessity of examining the behavior of pure PE liposomes.

Effect of T_H on the Destabilization of PE Liposomes. In order to fully comprehend the correlation between the destabilization of PE liposomes and the T_H of PE, we monitored the temperature dependence of H^+- and Ca^{2+}-induced leakage, lipid mixing, and mixing of contents of the liposomes. We used three different PE's (DOPE, EPE, and TPE) in order to have relatively well-spaced T_H's, with midpoints at ~12, 47, and 59 °C, respectively.

Our results give a clear picture of the initial interaction between PE liposomes at and above the T_H following addition of H^+ or Ca^{2+}. Leakage rapidly increases both in rate and in extent, whereas the mixing of contents is inhibited. For fusion to occur, it is necessary that following the contact-induced destabilization a new bilayer is formed encapsulating the mixed contents. It may be that at or above T_H the lipids collapse rapidly into H_{II} phase like tubes starting from aggregation of inverted micelles (Siegel, 1984).

It is important to point out that the ultimate relevance of these results for the control of fusion in cellular systems may lie in the destabilization of the apposed membranes, which is obviously enhanced at and above T_H. Other membrane components may restrict the extensive formation of nonbilayer structures and thereby promote, as a separate step, the subsequent communication of aqueous contents.

PE and Fusion below T_H. It is quite interesting that below the T_H, both H^+ and Ca^{2+} can induce some mixing of aqueous contents. The ability of Ca^{2+}, as well as other divalent and trivalent cations, to induce the fusion (mixing of aqueous contents) of acidic phospholipid liposomes is well-known

[reviewed by Nir et al. (1983)]. PS SUV eventually transform to large lamellar sheets at pH 3.5 (Papahadjopoulos et al., 1977), and we have recently shown mixing of aqueous contents for PS LUV at pH 2.0 (Ellens et al., 1985b). The different pH thresholds mainly reflect the kinetics of the process in that the earlier study used much larger lipid concentrations and monitored the transformation after 2 h. Now we find with PE, a negatively charged phospholipid at pH 9.5, introducing H^+ or Ca^{2+} can produce fusion below T_H, where the PE remains in the lamellar phase throughout the process.

While the extent of aqueous contents mixing is not large, due probably to the multilamellarity of the liposomes, this fusion below T_H suggests a simple phenomenological correlation between the H^+-induced fusion of PE and PS liposomes, as well as between the Ca^{2+}-induced fusion of PE and PG liposomes.

For the low-pH-induced fusion of PS (pH ~2.0) and PE (pH ~4.5), we note that the PS head group is $-(PO_4^-)$-$CH_2CH(NH_3^+)COOH$, whereas the PE head group is $-(PO_4^-)CH_2CH_2(NH_3^+)$. Both head groups are zwitterionic with the phosphate and amine groups. The difference is the protonated carboxyl group on PS. Whatever the intermolecular forces which drive the PE liposome fusion, it is quite likely that the PS head group will respond to the same forces. It may be relevant that below T_H the low-pH equilibrium morphologies for PE (Verkleij, 1984) and PS (Paphadjopoulos et al., 1977) are extensive flat sheets. For PS in Ca^{2+}, cochleates are found, and in Mg^{2+}, multilamellar liposomes are found (Papahadjopoulos et al., 1977). Discussions of the mechanism of PE liposome aggregation may be germane to the fusion mechanism (Kolber & Haynes, 1979; Rand, 1981).

The fusion of PG LUV requires in excess of 10 mM Ca^{2+} in 100 mM Na^+ buffer (Rosenberg et al., 1983), which from the Ca^{2+}/PG binding constant (Lau et al., 1981) implies about ~0.4 Ca bound/PG head group [calculated by using the equations given in Bentz (1981)]. A similarly large amount of bound Ca^{2+} is needed to induce the fusion of the PE liposomes (Figure 9) as calculated by using the Ca^{2+}/PE headgroup binding constant of 3 M^{-1} (McLaughlin et al., 1981). It can be presumed that Ca^{2+} binds to the phosphate group for both PG (Lau et al., 1981) and PE, at pH 9.5, since there has not been any evidence of Ca^{2+}/NH_2 binding for these types of molecules (Sigel & Martin, 1982). With Ca^{2+} bound to the phosphates of both PG and PE (pH 9.5), the head groups are quite similar and contain no other charged groups. The fusion of these liposomes is quite different from the Ca^{2+}-induced fusion of PS LUV, where only about 0.15 Ca bound/PS head group is required to induce fusion (Bentz et al., 1983b; Bentz & Düzgüneş, 1985).

Role of Interbilayer Contact in Inducing Destabilization. Implicit throughout this discussion is that interbilayer contact is the starting point of destabilization. While lipid mixing or fusion obviously requires contact, leakage may not. As mentioned above, we have shown for the H^+-induced destabilization of CHEMS/PE liposomes that leakage begins only after contact (Ellens et al., 1984). In this study, we have found that the PE liposomes destabilize upon contact and that there is an additional first-order kinetic process which leads to leakage (Figure 11). Our explanation for this first-order process is that oligomeric liposomes can collapse following the contact of the bilayers within each liposome.

This point is important for elucidating the molecular changes which lead to the destabilization. Cullis et al. (1980) pointed out that inverted micelles or lipidic particles could arise either from apposed exterior monolayers or within one bilayer. Siegel (1984) provided a theoretical analysis which indicated that lipidic particles would be much more rapidly formed from apposed exterior monolayers than within one bilayer. We know that liposome contact can induce leakage, which is sharply enhanced at the T_H of the lipid. Thus, the apposed monolayers can clearly promote the formation of the nonbilayer structures which begin the lamellar–H_{II} phase transition. If the additional first-order leakage kinetics we see are really due only to bilayer contact within the oligomeric liposomes, then the apposed monolayers are required for the onset of the transition.

The PE liposomes containing 0.1 mol % of the fluorophores NBD-PE and Rh-PE show an increase in fluorescence in the hexagonal phase transition temperature range. Since the probes are already maximally separated, this means that at and above T_H, there is an increase in the quantum efficiency of NBD-PE as the lipids go from a lamellar to a hexagonal phase. This may provide a new and very sensitive technique for measuring the T_H at very low lipid concentrations. The increase in fluorescence of the 0.1% NBD/Rh liposomes is only observed at and above the hexagonal phase transition temperature range at pH 4.5 or at pH 9.5 in the presence of Ca^{2+}. There is no change in fluorescence when the liposomes are incubated at high temperatures at pH 9.5.

For all cases shown in Figures 5 and 6, the rate of increase in fluorescence of the 0.1% NBD/Rh and the 0.1% NBD liposomes at and above T_H is dependent on the liposome concentration (Figure 11). The requirement of bilayer contact for the enhancement of the NBD quantum efficiency, to the extent proven, is the best evidence available that the morphological changes involved with the lamellar–H_{II} phase transition, which the NBD probe is monitoring, begin essentially upon liposome contact. Caffrey (1985) has found this phase transition to occur within 2 s for bulk lipid samples.

Conclusions

In this study, we have shown that the H^+- and Ca^{2+}-induced destabilization of PE liposomes involves liposome–liposome contact and responds to T_H. Bilayer destabilization is enhanced dramatically when the temperature approaches the T_H of DOPE, EPE, and TPE liposomes, mainly resulting in a massive enhancement of leakage, while fusion is diminished or abolished. As discussed in Bentz et al. (1985b), since the T_H is an equilibrium property involving a large ensemble of lipid molecules and the destabilization of two liposomes involves only a small number of molecules in a nonequilibrium event, this correlation is not necessarily expected. While the different morphologies accessible to PE at or above T_H can be very effective in inducing the initial destabilization of two apposing membranes, additional factors are required for fusion, defined as the concomitant mixing of bilayers and aqueous contents.

Acknowledgments

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Registry No. DOPE, 2462-63-7; H^+, 12408-02-5; Ca, 7440-70-2.

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