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On the correlation between H_{II} phase and the contact-induced destabilization of phosphatidylethanolamine-containing membranes

(liposome/H^+-induced fusion/hexagonal-phase transition/fluorescence energy transfer)

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ABSTRACT **The abundance of phosphatidylethanolamine (PtdEtn) in biological membranes and the capacity of this lipid to sustain nonbilayer structures have been promoted as evidence for a role of PtdEtn in biological fusion processes. To date there has been no direct evidence of a connection between the kinetics of bilayer destabilization and the polymorphism accessible to PtdEtn. We have developed a model system to examine this point directly using the proton-induced destabilization of PtdEtn/cholesterylhemisuccinate unilamellar liposomes. We find that the initial rate of bilayer mixing rapidly increases with temperature and reaches a maximal level just below the H_{II}-phase transition temperature. The leakage from these liposomes rapidly increases, both in rate and extent, within the H_{II}-phase transition temperature range. Of an even greater significance is that at no temperature is there any mixing of aqueous contents within the liposomes. Thus, these lipids can begin to undergo the lamellar- to H_{II}-phase transition at the stage of two apposed liposomes. However, the nonbilayer structures formed do not cause fusion—i.e., the concomitant mixing of aqueous contents.**

Although the amphipathic lipids found in biological membranes are normally considered to be in a bilayer structure, it is well known that when dispersed in pure form there is a group of these lipids that present nonbilayer structures. The most well-examined class of this group is phosphatidylethanolamine (PtdEtn) (1). Depending upon the acyl chain composition of the PtdEtn, there is a specific temperature range below which an aqueous dispersion of the lipid exists in the bilayer or lamellar phase and above which it exists in the hexagonal H_{II} phase. This temperature range is denoted T_H. Intermediate between these two thermodynamic phases are the lipidic particles (1). Though the molecular architecture underlying the electron microscopically observed lipidic particles is debatable—i.e., whether they are inverted micelles (1–4) or other nonbilayer configurations (5–7)—there is a general agreement that the ability of PtdEtn to form these nonbilayer structures is relevant to the fusion event between two apposed membranes (1–9). As yet, there has never been any direct evidence to support this claim.

The essential point to recognize here is that there does not need to be a direct correlation between an equilibrium state and the kinetic pathway leading to this state. The measurement of the lamellar- to H_{II}-phase transition temperature or the observation of lipidic particles is a thermodynamic event involving a large ensemble of lipid molecules. The interaction of two liposomes that leads to their mutual destabilization and leakage involves a small number of molecules and is a nonequilibrium event. It is the *first* step of the pathway. Even the nonbilayer structures captured via rapid-freezing techniques (1) may bear no resemblance to the nonbilayer structures that mediate the initial fusion event.

With this in mind, we can ask the most basic question: Is there a correlation between the T_H of a PtdEtn-containing lipid dispersion and the initial kinetics of bilayer destabilization between liposomes with this composition? This is the crux of the issue as to whether the T_H has a role in fusion occurring in biological systems and it has never been addressed experimentally.

The evidence to date is that PtdEtn's polymorphism is not required for the initial destabilization and fusion of membranes. It is well known that liposomes composed of lipids that do not show this polymorphism will fuse, most notable being the Ca^{2+}-induced fusion of phosphatidylserine (PtdSer)-containing liposomes (10, 11). Likewise, the Ca^{2+}-induced fusion of PtdSer/PtdEtn liposomes will occur well below the T_H of the lipid mixture (12, 13). In fact, the Ca^{2+}-induced fusion of cardiolipin/phosphatidylcholine liposomes shows no initial evidence of lipidic particles; however, in time and after much fusion, the lipidic particles emerge (14). Nonetheless, bilayer fusion necessarily implies the existence of nonbilayer structures. Hence, with PtdEtn-containing liposomes it is impossible to exclude a few transient inverted micelles as fusion intermediates between the apposed bilayers (1, 9, 14).

To address this question, we have used unilamellar liposomes (diameter, ≈ 0.1 μm) composed of a PtdEtn obtained by transesterification of egg phosphatidylcholine (tPtdEtn) and a cholesteryl ester, cholesterylhemisuccinate (Chol-Suc), which is negatively charged at neutral pH (15) (see Fig. 1 legend for more details). The charge on the Chol-Suc stabilizes the tPtdEtn in a liposomal bilayer. When these liposomes are placed into a low pH buffer, pH <5.5, the hemisuccinate group is protonated, which leads to aggregation of the liposomes and leakage of their contents (15). We have shown that these liposomes require interbilayer contact before they leak. Thus, even after protonation, where the bilayers are essentially composed of tPtdEtn and cholesterol, which is not stable as a unilamellar liposome, the contact of another liposome is required to produce leakage (15). We show here that when the temperature increases to the T_H range, there is a large enhancement of lipid mixing and leakage. However, at no temperature is there any fusion—i.e., mixing of aqueous contents.

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Abbreviations: PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; tPtdEtn, PtdEtn obtained by transesterification of egg phosphatidylcholine; Chol-Suc, cholesterylhemisuccinate; NBD-PtdEtn, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PtdEtn; Rh-PtdEtn, *N*-(lissamine rhodamine B sulfonyl)-PtdEtn; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; ANTS, 1-aminonaphthalene 3,6,8-trisulfonic acid; DPX, *p*-xylylenebis(pyridinium bromide); DSC, differential scanning calorimetry.

MATERIALS AND METHODS

In all experiments the liposomes were prepared by the reverse phase evaporation method (15, 16). For the differential scanning calorimetry (DSC) scans the liposomes were prepared in 25 mM histidine/25 mM 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid (Tes)/100 mM NaCl/0.1 mM EDTA, pH 7.4, and were extruded through polycarbonate membranes with 0.2-μm pores (Nuclepore). Total lipid concentration was 50 μmol/ml. One milliliter of the liposome suspension was dialyzed against 250 ml of 50 mM acetate/acetic acid and 110 mM NaCl buffer (pH 4.5) for 1 hr. The intraliposomal buffer and the dialysate were isoosmotic. The liposomal suspension was then concentrated in an Eppendorf centrifuge (12,800 × *g* for 1 min) and the pellet was dispersed in 50 μl of the dialysate buffer. Seventeen microliters of this suspension was sealed in an aluminum sample pan and the DSC scan was performed by using a Perkin–Elmer DSC-2 calorimeter operating at a sensitivity of 1 mcal/sec (1 cal = 4.184 J) and at a scanning rate of 5°C/min. tPtdEtn was obtained from Avanti Polar Lipids (Birmingham, AL) and Chol-Suc was obtained from Sigma (15).

Lipid mixing was monitored by the method of Struck *et al.* (17). One part of labeled liposomes [containing 1 mol% each of *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PtdEtn (NBD-PtdEtn) and *N*-(lissamine rhodamine B sulfonyl)-PtdEtn (Rh-PtdEtn), both purchased from Avanti Polar Lipids] was mixed with nine parts of unlabeled liposomes. NBD-PtdEtn fluorescence is quenched by Rh-PtdEtn due to Förster energy transfer (17). Bilayer fusion leads to lipid probe dilution and the increase of NBD-PtdEtn fluorescence. The assay was calibrated with 0% fluorescence set with the liposomes at pH 7.4, where there was no lipid mixing, and 100% fluorescence was set with 50 μM tPtdEtn/Chol-Suc liposomes containing 0.1 mol% each of NBD-PtdEtn and Rh-PtdEtn and otherwise was identical to the labeled liposomes, which would correspond to complete lipid mixing (18). These calibration levels were insensitive to pH between 4.5 and 7.4. At and above T_H, the fluorescence signal from lipid mixing eventually exceeded 100%, reaching 130% for the tPtdEtn/Chol-Suc 8:2 liposomes at the highest temperature. Evidently, the conversion of the lipid to the H_{II} phase alters the quantum efficiency of the NBD fluorescence. This effect requires further analysis, possibly as a probe for H_{II}-phase formation. Since this "extra" fluorescence may depend upon other unknown factors, we chose to use the 100% obtained by the 0.1% liposomes at pH 7.4. Fluorescence was measured on an SLM-4000 fluorometer with a magnetically stirred 1-ml cuvette. Excitation was at 450 nm and emission was measured at 530 nm. For these experiments the liposomes were made in 100 mM NaCl/2 mM Tes/2 mM histidine/0.1 mM EDTA, pH 7.4, and extruded through 0.1-μm Nuclepore filters (15, 18).

Leakage was measured by the release of the pH-insensitive fluorophore 1-aminonaphthalene 3,6,8-trisulfonic acid (ANTS) from the liposomes into the medium. ANTS fluorescence in the liposomes is quenched by collisional energy transfer to *p*-xylylenebis(pyridinium bromide) (DPX) (15, 18). Leakage was calibrated with 0% set to the residual fluorescence of the liposomes at pH 7.4, where there is no leakage, and with 100% set to the fluorescence obtained after lysis of the liposomes with Triton X-100 at pH 4.5. We have shown before that leakage of contents equals the change in fluorescence (15). We have also used this assay to monitor the mixing of aqueous contents between fusing liposomes by encapsulating ANTS in one population of liposomes and DPX in another (18). Fusion results in a quenching of ANTS fluorescence. With this assay, we have found no mixing of aqueous contents between the tPtdEtn/Chol-Suc liposomes at either molar ratio or at any temperature. On the other hand, Ca^{2+} or Mg^{2+} will induce the mixing of aqueous contents of these liposomes at temperatures well below the T_H (18). Hence, with H^+ the liposomes show lipid mixing and leakage but not mixing of aqueous contents. The liposomes were prepared by extrusion through a 0.1-μm Nuclepore filter and contained either 12.5 mM ANTS/45 mM DPX/20 mM NaCl for the leakage experiments or 25 mM ANTS/40 mM NaCl and 90 mM DPX for the fusion experiments. The aqueous contents were buffered with 10 mM Tris·HCl at pH 7.5. The intraliposomal contents and the pH 4.5 buffer were all isoosmotic. Other conditions were identical to those in the lipid-mixing experiment.

RESULTS AND DISCUSSION

Fig. 1 shows the DSC scan of tPtdEtn/Chol-Suc liposomes in an 8:2 and 7:3 molar ratio at pH 4.5. For the 8:2 liposomes there was a gel–liquid crystalline-phase transition in the range of 0–10°C and a lamellar–H_{II}-phase transition in the range of 36–44°C. For the 7:3 liposomes the gel–liquid crystalline-phase transition was abolished and the lamellar–H_{II}-phase transition was decreased to 23–37°C. Hence, increasing the proportion of Chol-Suc decreased the lamellar–H_{II}-phase transition temperature, as has been noted for PtdEtn/cholesterol mixtures (20, 21). By way of reference, for pure tPtdEtn the gel–liquid crystalline-phase transition occurred in the range of 0–17°C and the lamellar–H_{II}-phase transition occurred in the range of 55–60°C (data not shown).

In Fig. 2 we show the kinetics of bilayer mixing for both the 7:3 and 8:2 tPtdEtn/Chol-Suc liposomes as a function of temperature. The fluorescence assay for monitoring lipid mixing, developed by Struck *et al.* (17), is described in the figure legend. In all cases, bilayer contact was required for probe exchange to occur. Fig. 3 shows the kinetics of leakage of contents from the same sets of liposomes over the same temperature range (see legend for details). We also determined that there was no mixing of aqueous contents between these liposomes at any temperature (see Fig. 3 legend for details). Thus, at pH 4.5, the tPtdEtn/Chol-Suc liposomes aggregated at all temperatures (result not shown) and there was bilayer destabilization—i.e., lipid mixing and leakage of contents; however, there was no fusion of the liposomal contents.

Fig. 4 shows the initial rates of lipid mixing between and leakage from these liposomes as a function of temperature.

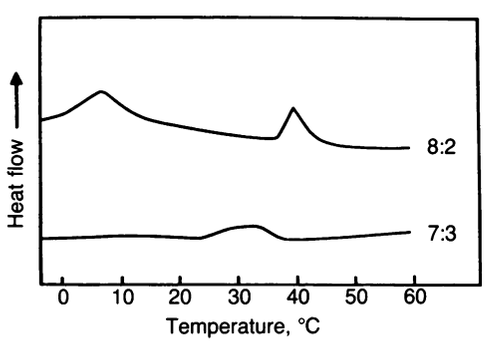

FIG. 1. DSC scans of tPtdEtn/Chol-Suc liposomes (diameter, ≈0.2 μm) with an 8:2 and 7:3 molar ratio at pH 4.5. For the 8:2 liposomes the main transition, gel–liquid crystalline, occurs between 0°C and 10°C, whereas the lamellar–H_{II}-phase transition occurs between 36°C and 44°C. For the 7:3 liposomes the gel–liquid crystalline transition is abolished, whereas the lamellar–H_{II}-phase transition is between 23°C and 37°C. These peaks were unchanged after repeated scans. The T_H range is sensitive to pH (19, 20)—i.e., in our case, the extent of Chol-Suc protonation. At pH 7.4 for either tPtdEtn/Chol-Suc molar ratio the T_H exceeds 70°C.

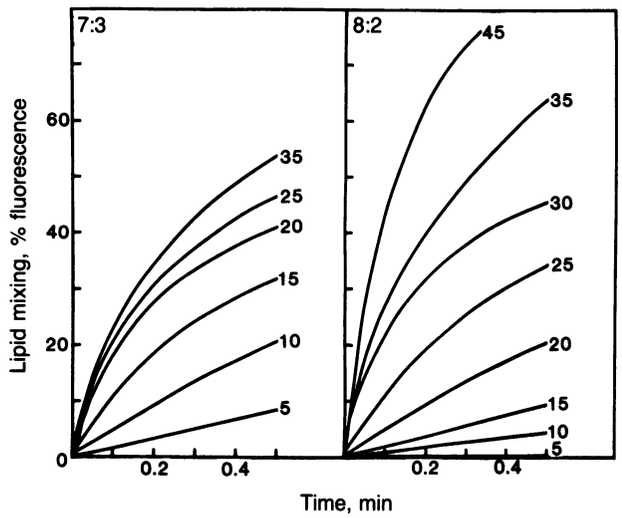

FIG. 2. Kinetics of H^+-induced lipid mixing of tPtdEtn/Chol-Suc liposomes (diameter, ≈0.1 μm) at 7:3 and 8:2 molar ratios shown as a function of temperature, which is given to the right of each curve in °C. Liposomes were injected into a pH 4.5 buffer (acetate/acetic acid and isoosomotic with the intraliposomal contents) and the extent of lipid mixing due to bilayer fusion is shown as a function of time. Lipid mixing was monitored by the method of Struck *et al.* (17) by using one part of liposomes containing 1 mol% each of NBD-PtdEtn and Rh-PtdEtn and nine parts of unlabeled liposomes (final total lipid concentration, 50 μM). The bilayer fusion results in a relief of quenching of NBD-PtdEtn fluorescence due to the dilution of its quencher Rh-PtdEtn into the unlabeled lipid pool. The curves show this fluorescence increase. It is known that this increase in fluorescence is not a linear function of the lipid mixing (17); however, the fluorescence curves can only underestimate the extent of lipid mixing, even at lower NBD/Rh mol% values. Since fluorescence intensity increases monotonically with lipid mixing, it is clear that lipid mixing is enhanced dramatically at about 10°C below the T_H. The change in NBD fluorescence quantum efficiency, in the equilibrium H_{II} phase, will have little effect on this conclusion since the increase starts below T_H.

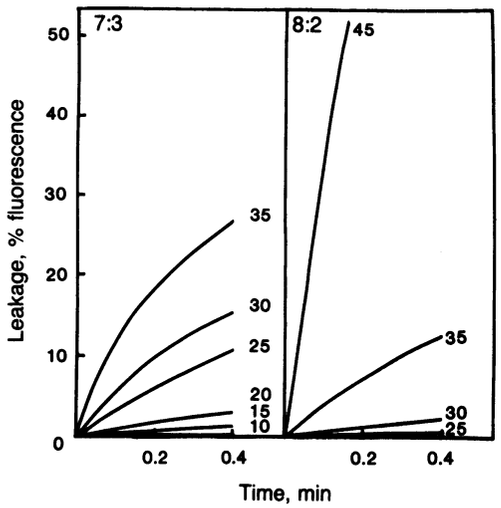

FIG. 3. Kinetics of H^+-induced leakage from tPtdEtn/Chol-Suc liposomes (diameter, ≈0.1 μm) at 7:3 and 8:2 molar ratios shown as a function of temperature, which is given to the right of each curve in °C. Liposomes were injected into the pH 4.5 buffer. Leakage was measured by the release of coencapsulated ANTS/DPX, which results in the relief of ANTS quenching by DPX (15). The curves show the increase in fluorescence, which we have shown previously to be essentially equal to the leakage of contents (15). By encapsulating ANTS in one population of liposomes and DPX in the other we have shown that the mixing of aqueous contents within fusing liposomes can be monitored by this assay by using the Ca^{2+}-induced fusion of PtdSer liposomes [where it matched the results of the terbium/dipicolinic acid assay (18)] and the Ca^{2+}- or Mg^{2+}-induced fusion of tPtdEtn/Chol-Suc liposomes identical to those used here (18). Here, using the ANTS/DPX system as a fusion assay, we find that with H^+ there is no mixing of aqueous contents within the liposomes at any temperature for either tPtdEtn/Chol-Suc molar ratio (data not shown). For all experiments here the final total lipid concentration was 50 μM.

For convenience, the lamellar–H_{II}-phase transition range is shown by the horizontal arrows marked T_H. For the 8:2 tPtdEtn/Chol-Suc liposomes the gel–liquid crystalline-phase transition range is shown by the horizontal arrow marked T_C.

Taken together, these data provide a very clear picture of the effect of T_H on the initial interactions between two liposomes. Lipid mixing increases rapidly 10–20°C below T_H and the initial rate appears to level off at the T_H. Leakage increases rapidly in rate and extent within the T_H. Clearly, there are two mechanisms of destabilization. Below T_H the bilayers become apposed and limited destabilization occurs between the external monolayers, which accounts for the observed lipid mixing and low levels of leakage. At temperatures within or above the T_H the second mechanism—i.e., the extensive formation of inverted micelles or intermembrane attachment sites, perhaps leading to local H_{II} domains—leads to complete bilayer destabilization and leakage.

This result shows that the lamellar–hexagonal H_{II}-phase transition can begin at the stage of two apposed liposomes. Thus, the molecular mechanism of bilayer destabilization at and above T_H is the appearance of the inverted micelles or lipidic particles (1–9). Well below T_H, we do find slow lipid mixing and leakage. The mechanism of this destabilization is most likely the collapse of the aggregated liposomes to lamellar sheets, which is the equilibrium state. Liposomes composed of pure PtdEtn have only been made at high pH, ≥9.0, where the PtdEtn head group is negatively charged (22, 23). Putting these liposomes into lower pH buffers results in aggregation and lipid mixing (24).

With this study we have shown that there is, in fact, a direct correlation between the T_H measured at equilibrium for an H_{II}-phase-competent lipid system and the initial destabiliza-

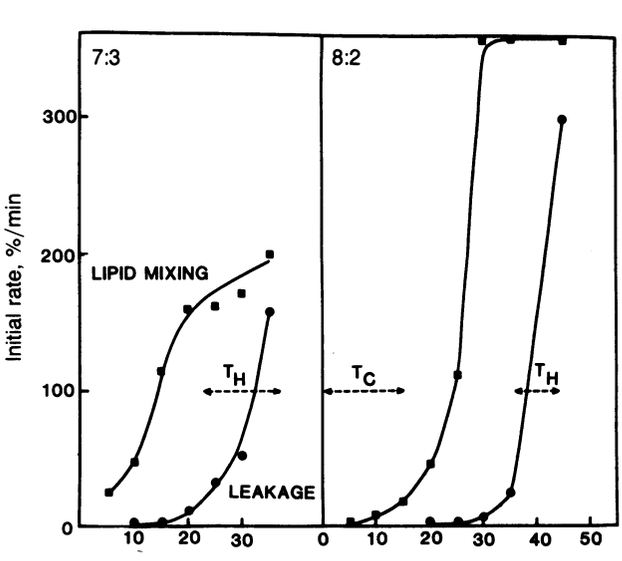

FIG. 4. The initial rates of lipid mixing (■) and leakage (●) for the tPtdEtn/Chol-Suc liposomes at 7:3 and 8:2 molar ratios shown as a function of temperature. The initial rates are just the initial slopes of the curves shown in Figs. 2 and 3 in the units of %/min. The dashed arrow denoted T_H shows the lamellar–H_{II}-phase transition temperature range, whereas the dashed arrow denoted T_C shows the gel–liquid crystalline-phase transition temperature range for the 8:2 tPtdEtn/Chol-Suc liposomes.

tion of the liposomes. However, the contact-induced destabilization within or above T_H does not lead to any mixing of aqueous contents. This is an important distinction. Though we have proven here that the pre-H_{II}-phase structures can arise between two apposed membranes, it has always been the speculation that if these structures form, then they should lead to some mixing of aqueous contents, as required of a model for biological fusion (1–9). The polymorphism accessible to PtdEtn-containing membranes can be a powerful agent for inducing the initial destabilization of the apposed bilayers, but for fusion additional factors (25) are required.

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