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## pH-Induced Destabilization of Phosphatidylethanolamine-Containing Liposomes: Role of Bilayer Contact<sup>†</sup>

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**ABSTRACT:** The mechanism of pH-induced destabilization of liposomes composed of phosphatidylethanolamine and a charged cholesterol ester was studied by following the release of encapsulated aqueous contents. The kinetics of release were measured continuously by using the water-soluble fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid in combination with the water-soluble quencher *p*-xylylenebis(pyridinium) bromide. With this fluorescence assay, release of contents from liposomes composed of phosphatidylethanolamine and cholesterol hemisuccinate was shown to be a function of pH, ratio of phosphatidylethanolamine to cholesterol hemisuccinate, and acyl chain composition of the phosphatidylethanolamine.

**B**ilayer destabilization has been shown to require interbilayer contact in a large number of model membrane systems. This prerequisite for membrane fusion has been shown for liposomes composed of acidic phospholipids in the presence of divalent cations (Wilschut et al., 1980; Liao & Prestegard, 1980a,b; Nir et al., 1983; Bentz et al., 1983b) and liposomes composed of saturated phosphatidylcholines below the isothermal phase transition temperature (Suurkuusk et al., 1976; Schullery et al., 1980; Wong et al., 1982). It has been proposed that this is also the case with phosphatidylethanolamine (PE)<sup>1</sup>-containing bilayers (Cullis & Hope, 1978; Cullis et al., 1980; Verkleij et al., 1980; Mantsch et al., 1981) but this has not yet been proven.

Pure PE liposomes can only be made above pH 9.0 (Stollery & Vail, 1977), where the PE is negatively charged. Injection of these liposomes into pH 6.0 buffers, where the PE is protonated, leads to aggregation, leakage, and lipid mixing (Kolber & Haynes, 1979; Pryor et al., 1983). These studies, however, did not show that aggregation, or bilayer contact, was necessary for bilayer destabilization and leakage, since the possibility that leakage occurred independently of aggregation could not be ruled out. Moreover, the protonation of the PE may promote other molecular rearrangements and bilayer perturbations that do not pertain to the interactions between PE-containing bilayers at physiological pH.

Stable PE-containing liposomes can be formed at physiological pH when PE is mixed with acidic phospholipids, due

Leakage was very slow at pH 5.5 and increased dramatically with decreasing pH down to 4.0. Replacing phosphatidylethanolamine by phosphatidylcholine eliminated the effect of pH on leakage. Analysis of the kinetics of release by a mass action model demonstrated that bilayer destabilization and leakage occur subsequent to aggregation. The requirement of bilayer contact for destabilization has been found previously for acidic phospholipid bilayers in the presence of divalent cation and for saturated phosphatidylcholine bilayers below the isothermal phase transition temperature. The phosphatidylethanolamine-containing bilayers examined here satisfy the same requirement.

to the stabilization by the negatively charged head groups. Mixing these liposomes with Ca<sup>2+</sup> (Düzgüneş et al., 1981; Sundler et al., 1981; Hope et al., 1983) or a pH 3.0 buffer (Hope et al., 1983) induces aggregation, destabilization, and fusion of the liposomes. However, neither of these systems is optimal for elucidating the role of PE in bilayer destabilization. With Ca<sup>2+</sup> the destabilization is dominated by interaction of the acidic phospholipids with the divalent cation. At the pH values necessary to protonate the acidic phospholipids (2.0–3.0), thus allowing aggregation of the liposomes, the further protonation of the PE cannot be excluded. In addition, it is difficult to devise assay systems that are competent over such wide pH ranges.

We have designed a lipid system that can assess the importance of membrane contact in bilayer destabilization of PE-containing liposomes at physiological pH. Stable liposomes can be made with PE and cholesterol hemisuccinate (CHEMS) at pH 7.5, where the CHEMS is negatively charged. When the CHEMS is protonated, below pH 5.5, the liposome is effectively composed of PE and cholesterol. By inducing the destabilization of the liposomes by H<sup>+</sup> at physiological pH values (4.5–7.5), one can study the effect of contact per se, since the PE remains zwitterionic in this range. To investigate the mechanism of pH-induced release of liposome contents, we developed a quantitative assay that allows for leakage to be monitored at very early times and continu-

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<sup>1</sup> Abbreviations: PC, egg phosphatidylcholine; TPE, phosphatidylethanolamine prepared from egg PC by transesterification; PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; CHEMS, cholesterol hemisuccinate; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylylenebis(pyridinium) bromide; Tris, tris(hydroxymethyl)amino-methane hydrochloride.

ously. Fluorescent markers, e.g., carboxyfluorescein (Weinstein et al., 1977), calcein (Allen & Cleland, 1980), and terbium/dipicolinic acid (Wilschut & Papahadjopoulos, 1979) have proven to be very useful in the continuous monitoring of leakage (carboxyfluorescein and calcein) and mixing of aqueous contents (terbium/dipicolinic acid). However, these fluorophores are themselves very sensitive to pH. Most of their fluorescence is extinguished by proton titration below pH 5.0 (Barela & Sherry, 1976; Szoka et al., 1979). To avoid this problem we used a different assay system, involving the fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), which is relatively insensitive to pH above 4.0 and which can be quenched by *p*-xylylenebis(pyridinium) bromide (DPX) (Smolarsky et al., 1977).

Through the use of the ANTS/DPX assay and the PE/CHEMS lipid system we were able to elucidate the mechanism of pH-induced release. When incubated in pH 5.0–4.0 buffers, liposomes composed of TPE (transesterified from PC) or DOPE and CHEMS aggregate and release their contents. When the pH-induced leakage was analyzed by a mass action kinetic model, it was found that pH-induced leakage occurs after liposome aggregation, i.e., liposome aggregation and close apposition of the bilayers is necessary for leakage. Evidently, TPE or DOPE and CHEMS are stable in a liposomal bilayer configuration even at low pH where CHEMS is protonated, but only *until* the liposomes aggregate, and the close apposition of the bilayers induces the destabilization. This system and analysis are the first to clearly discriminate between pH-induced aggregation and leakage.

#### Materials and Methods

Egg phosphatidylcholine (PC), dioleoylphosphatidylethanolamine (DOPE), and egg phosphatidylethanolamine, prepared by transesterification from egg phosphatidylcholine (TPE), were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesteryl hemisuccinate (CHEMS) was purchased from Sigma. 8-Aminonaphthalene-1,3,6-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.

In all cases the liposomes were prepared by reverse-phase evaporation according to Szoka & Papahadjopoulos (1978). The liposomes were prepared with solutions containing ANTS and DPX (at various molarities as specified), buffered with 10 mM Tris [tris(hydroxymethyl)aminomethane hydrochloride] at pH 7.5. The lipid (45  $\mu$ mol) was dissolved in 6 mL of ether (stored over distilled, deionized water; Sybron/Barnstead, Boston, MA) and sonicated for 5 min in a bath-type sonicator under nitrogen with 2 mL of the aqueous phase. The resulting emulsion was evaporated in a rotary evaporator at room temperature under reduced pressure (400 mmHg) to remove ether. After collapse of the gel, evaporation was continued under high vacuum (150 mmHg) for 15–20 min to remove residual ether. The liposomes were extruded through polycarbonate membranes (Bio-Rad) with 0.1- $\mu$ m pores under a nitrogen pressure of approximately 80 psi (Olson et al., 1979). The liposomes were separated from nonencapsulated material on Sephadex G-75 (Pharmacia) equilibrated with Tris buffer (50 mM Tris,  $\sim$ 100 mM NaCl, pH 7.5; equiosmotic to the encapsulated solution). The encapsulated volume was 1.2  $\mu$ L/ $\mu$ mol of total lipid. The lipid concentrations were determined by lipid phosphorus assay according to Bartlett (1959).

Coencapsulation of DPX with ANTS extinguishes most of the ANTS fluorescence as described below (Smolarsky et al., 1977). Leakage of ANTS from the liposomes could be followed by the increase in fluorescence due to the relief of DPX quenching.

We determined that there was no significant binding of ANTS to the liposomes by incubating "empty" TPE/CHEMS, 7/3, liposomes (containing Tris/NaCl buffer, pH 7.5) with a solution containing 50 mM ANTS, 50 mM DPX, and 10 mM Tris, pH 7.5, for 30 min at room temperature. The medium was separated from the liposomes on Sephadex G-75 and the remaining fluorescence associated with the liposomes was  $<0.5\%$  of the total fluorescence that the liposomes would have encapsulated. When these liposomes were lysed with 0.5% Triton X-100 or incubated in an acetate buffer at pH 4.5, the fluorescence level did not change. Therefore, under the conditions of the assay, ANTS is a valid marker of the aqueous space.

Fluorescence and light scattering were measured simultaneously with an SLM 4000 fluorometer (SLM Instruments, Champaign, Urbana, IL) equipped with two 90° emission channels. Excitation was at 384 nm. Emission was measured through a Corning 3-68 cutoff filter ( $>530$  nm), which eliminated all but 0.2% of scattered light. Light scattering was measured in the second emission channel by using a Corning 7-54 band-pass filter. In the leakage experiments the residual fluorescence of the liposomes containing 50 mM DPX and 50 mM ANTS was taken as 0% release. Maximal fluorescence, obtained after disruption of the liposomes with Triton X-100 (0.5%) was taken as 100% release. It is shown below that the relative fluorescence measured at any time represents percent of leakage. Leakage was measured at 25 °C in buffers of different pH (50 mM Tris, pH 7.5; 50 mM sodium acetate/acetic acid, pH 5.5, 5.0, 4.5, and 4.0; all buffers contained  $\sim$ 100–150 mM NaCl to give an osmotic strength equal to the encapsulated ANTS/DPX solution). The leakage experiment was started by injection of small volumes (10–50  $\mu$ L, using a Hamilton syringe) of concentrated liposome suspensions into a magnetically stirred cuvette, containing 1 mL of the buffer. The results were recorded on an Omniscribe chart recorder, at rapid chart speeds when necessary.

#### Results

*A Fluorescence Method To Measure Leakage from Liposomes at Low pH.* The inset in Figure 1 shows that the ANTS fluorescence is relatively insensitive to pH, with only a 7% drop in relative fluorescence intensity between pH 7.5 and pH 4.5. This may be compared with a more than 90% drop in fluorescence for carboxyfluorescein between these pH values (Szoka et al., 1979).

ANTS does not self-quench since there is no significant overlap between its excitation and emission spectra (data not shown); however, ANTS fluorescence is efficiently quenched with DPX (Smolarsky et al., 1977). Figure 1 shows the quenching of ANTS by DPX. In solution, the same quenching curve was obtained with ANTS concentrations in the range of 0.01–1.0 mM. The quenching is dependent only upon the DPX concentration. This indicates that the quenching is due to classical Förster energy transfer and not to complex formation. It should be noted that ANTS fluorescence is subject to an inner filter effect at concentrations above approximately 0.1 mM; however, that does not affect the quenching by DPX. In addition, the quenching curves in solution are similar at pH 7.5 and 4.5.

Figure 1 also shows the quenching of ANTS by DPX in liposomes. We coencapsulated ANTS and DPX at 50, 25,

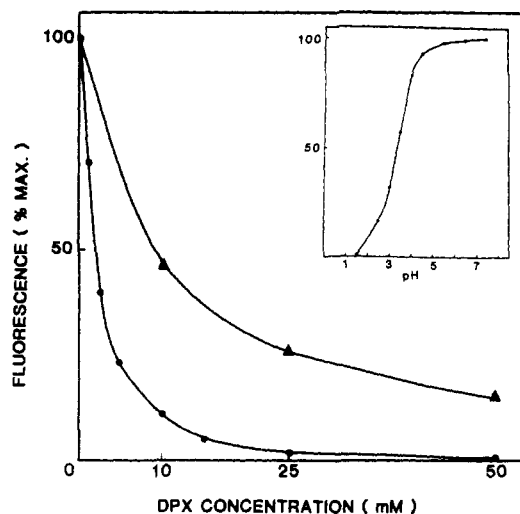


FIGURE 1: Quenching of ANTS fluorescence by DPX. The quenching of ANTS fluorescence by DPX in solution or in liposomes was measured at various DPX concentrations. For quenching in solution 100% fluorescence was set with 0.01 mM (or 1 mM) ANTS in Tris buffer, pH 7.5, and 0% fluorescence was set with Tris buffer (●). For quenching in liposomes ANTS and DPX were encapsulated at concentrations of 50, 25, and 10 mM of each. The residual ANTS fluorescence of the ANTS/DPX-containing liposomes was measured by using the total fluorescence of the liposomes lysed with 0.5% Triton X-100 to set 100% fluorescence and Tris buffer to set 0% fluorescence (▲). Inset: Effect of pH on ANTS fluorescence. The fluorescence of 0.01 mM ANTS was measured at various pH values. 100% fluorescence was set with 0.01 mM ANTS in Tris, pH 7.5; 0% fluorescence was set with buffer. ANTS fluorescence was measured in MES, pH 6.5, acetate/acetic acid, pH 5.5–3.5, glycine/HCl, pH 3.0 and 2.5, and HCl, pH 1.5.

and 10 mM of both and measured the quenching. It can be seen that the quenching of ANTS fluorescence by the DPX in liposomes is less efficient than in solution. However, for our experiments ANTS is sufficiently well quenched in liposomes (~85%) with the initial coencapsulation of 50 mM DPX. This quenching is completely relieved upon leakage of ANTS and DPX from the liposomes and dilution into the medium.

In order to be able to continuously monitor the kinetics of leakage, the release of ANTS quenching should be very fast, so that the fluorescence signal directly indicates the amount of leakage that has occurred. It takes less than 2 s from the time of injection of 5  $\mu$ L of a 25 mM ANTS/25 mM DPX solution into 1 mL of buffer, to exceed 95% dequenching (results not shown). This is the mixing time of the solutions in the cuvette, since the injection of just 5  $\mu$ L of 25 mM ANTS gave the identical curve for the increase in fluorescence. This dequenching reaction is fast enough to allow for a continuous measurement of leakage.

It is known that carboxyfluorescein leaks out of small unilamellar vesicles composed of phosphatidylcholine, PC, below pH 6.5, probably due to protonation of the fluorophore (Szoka et al., 1979). ANTS is very hydrophilic and contains three sulfonic acid groups with  $pK_a$  values between 0 and 1. These groups will be charged at pH 4.5, which reduces the diffusion of liposome-encapsulated ANTS across the intact bilayer at this pH. To verify this, we measured ANTS leakage from PC liposomes (extruded through polycarbonate membranes with 0.1  $\mu$ M pores) at low pH. The liposomes were incubated at 25  $\mu$ M liposomal lipid in a pH 4.5 acetate buffer. ANTS leakage amounted to only 0.05%/min (data not shown). This is negligible compared to leakage from the PE/CHEMS liposomes at this pH (results shown below). Hence, we conclude that ANTS does not become labile to the bilayer at or above

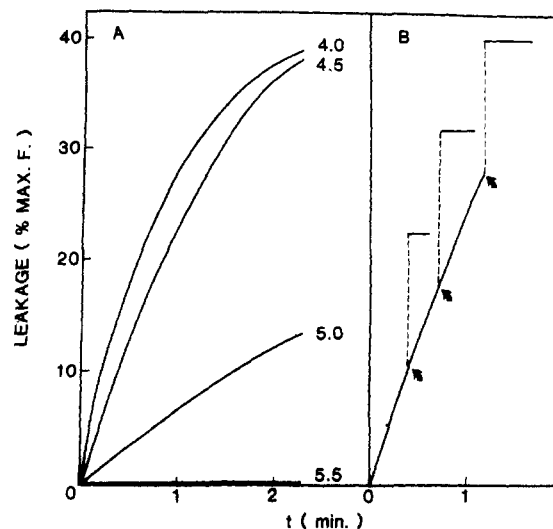


FIGURE 2: Effect of pH on TPE/CHEMS, 7/3, liposomes. 25  $\mu$ M TPE/CHEMS liposomes were injected into buffers of different pH, as indicated in the figure (50 mM sodium acetate/acetic acid, pH 5.5, 5.0, 4.5, and 4.0; ~150 mM NaCl to give the same osmotic strength as the encapsulated ANTS/DPX solution) (panel A). At various times after injection of the liposomes into the pH 4.5 buffer a small amount of NaOH was injected (arrow) to bring the pH back up to >6.0 (panel B). Fluorescence was measured as a function of time (see Materials and Methods).

pH 4.5. We now have available a combination of fluorophore and quencher that can be conveniently used to monitor leakage from liposomes at low pH.

**Effect of pH on TPE/CHEMS Liposomes.** We investigated the effect of pH on liposomes composed of TPE and CHEMS in a 7/3 molar ratio. The liposomes were injected into buffers with pH values between 7.5 and 4.0. Figure 2A shows that above pH 5.5, there is essentially no leakage. However, leakage begins between pH 5.5 and pH 5.0 and is accompanied by a decrease in light scattering, showing liposome aggregation (Düzgüneş et al., 1981). At lower pH, down to ~4.5, there is enhanced leakage and aggregation. Below pH 4.5, down to pH 4.0, the leakage rates do not change much. We believe that the aggregation and leakage is initiated by the protonation of the hemisuccinate on the CHEMS. The  $pK_a$  of the hemisuccinate group should lie in the region of 4.2–5.6, which are the two  $pK_a$ s of succinic acid.

As mentioned before (see Materials and Methods), the residual fluorescence of the liposomes at pH 7.5 is set to 0% release, and 100% release is set by lysing the liposomes with Triton X-100 at the final pH. The 7% drop in fluorescence between pH 7.5 (the initial intraliposomal pH) and pH 4.5 (the pH of the incubation medium) has only a negligible effect on our calibration, resulting in a maximum offset of only 1% of the calibrated fluorescence scale (7% of 15% residual liposomal fluorescence). The offset is even less when the final pH is higher.

We estimated the ratio of ANTS and DPX released from the liposomes at low pH. This was done by comparing the UV absorbance spectra of ANTS and DPX standards with that of the liposome incubation medium. TPE/CHEMS (7/3) liposomes (250  $\mu$ M) were incubated for 0.4 min at pH 4.5, then the pH was brought up to 6.0, and the medium was separated from the liposomes on Sephadex G-75. The results (data not shown) revealed that ANTS and DPX leak from the liposomes simultaneously. Furthermore, in similar experiments the total fluorescence intensity recovered from the column in the elution volume was essentially identical with that measured in the fluorometer (both for approximately 30%

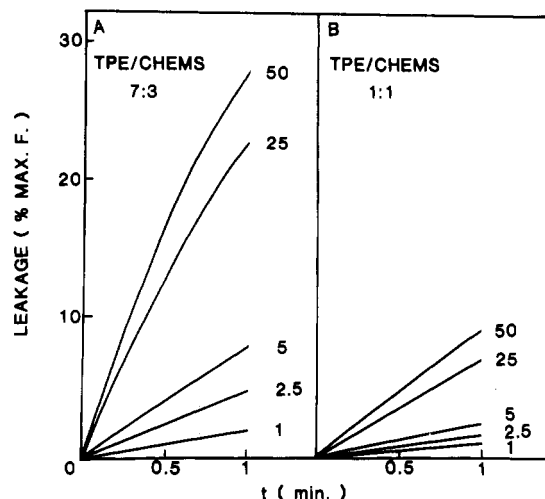


FIGURE 3: Effect of TPE/CHEMS ratio and liposome concentration on pH-induced leakage. Various concentrations (1–50  $\mu$ M liposomal lipid as indicated in the figure) of TPE/CHEMS, 7/3 (panel A), and TPE/CHEMS, 1/1 (panel B), liposomes were injected into pH 4.5 acetate buffer (50 mM sodium acetate/acetic acid,  $\sim$ 150 mM NaCl; equiosmotic to the encapsulated ANTS/DPX solution). Fluorescence was measured as a function of time (see Materials and Methods).

or 50% leakage), i.e., the relative fluorescence measured on the fluorometer is due only to ANTS outside the liposomes. These results imply that the fluorescence from the liposomes is constant at least up to 50% leakage. This result could be anticipated from the data in Figure 1.<sup>2</sup>

When the pH is increased from 4.5 to above 6, leakage stops after a few seconds (Figure 2B) and the aggregation is partially reversed, as evidenced by light scattering. In all cases, raising the pH led to a rapid release of 12–14% of the ANTS. The explanation for the constancy of this release is clearly not the pH dependence of the ANTS fluorescence (Figure 1). At present, we can only attribute this release to some combination of membrane reorganization following the deprotonation of the CHEMS and/or the possible existence of ANTS/DPX trapped in interliposomal spaces that escapes when the liposomes partially disaggregate.

<sup>2</sup> As ANTS and DPX leak out, the total fluorescence from liposome-encapsulated material does not increase initially. A numerical example will demonstrate this point. The liposomes initially containing 50 mM each of ANTS and DPX emit 15% of the fluorescence of the lysed liposomes (i.e., the ANTS is 85% quenched) and this fluorescence intensity is set to 0% leakage, while the fluorescence of the lysed liposomes is set to 100% leakage. With this calibration the relative fluorescence,  $F$ , is given by  $F = 100[(L + (1 - L)q - 0.15)/0.85]$ , where  $L$  is the fraction of ANTS leakage and  $q$  is the remaining fraction of fluorescence of the liposomes relative to the maximum fluorescence. The factor 0.15 arises from the initial 15% residual fluorescence of the intact liposomes and the denominator of 0.85 arises from normalizing this initial fluorescence to 100% leakage. Initially, when there is no leakage,  $L = 0$  and  $q = 0.15$ , since the liposomes have 15% fluorescence relative to the maximum fluorescence and  $F = 0$ . In this case,  $q$  is the fraction of residual fluorescence since all 50 mM of the ANTS is still encapsulated by the liposomes. When all of the ANTS has leaked, then  $L = 1$  and  $F = 100\%$ . Now at 50% leakage  $L = 0.5$  and the liposomes contain 25 mM of ANTS and DPX. From Figure 1 we find that the residual fluorescence of this mixture in liposomes is 26%. Since the residual fluorescence is defined relative to the encapsulated ANTS concentration (25 mM in this case), we have  $q = 0.13$ , i.e., half the fraction relative to 50 mM encapsulated ANTS. This implies that  $F = 48.8\%$  or just over a 1% underestimate of the true leakage. When 30% of the ANTS has leaked, then  $L = 0.3$ . The residual fluorescence of liposomes containing 35 mM each of ANTS and DPX is 20%, Figure 1; hence,  $q = 0.2(35/50) = 0.14$ . Thus,  $F = 29.2\%$ . Up to 50% leakage, the relative fluorescence,  $F$ , is within 2% of the true leakage of the liposomes. Of course these results only hold when the liposomes initially contain 50 mM ANTS and 50 mM DPX.

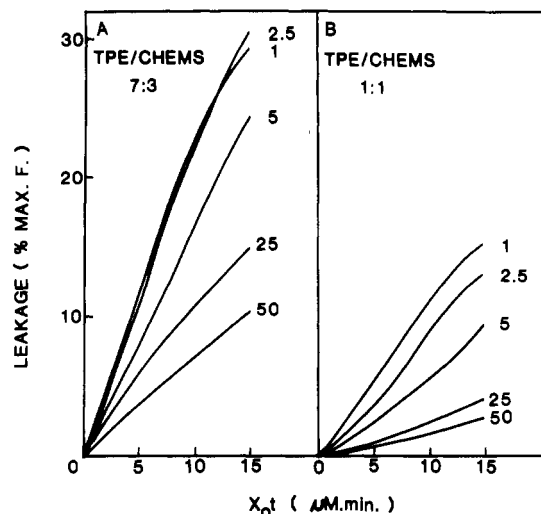


FIGURE 4: Relative leakage for various liposomal lipid concentrations of TPE/CHEMS, 7/3 (panel A), and TPE/CHEMS, 1/1 (panel B), liposomes in pH 4.5 buffer vs.  $X_0t$ , where  $X_0$  denotes the lipid concentration. The lipid concentrations (in  $\mu$ M) are indicated in the figure.

**Lipid Requirements for Destabilization.** We have seen that TPE/CHEMS liposomes leak in low pH, whereas PC liposomes do not leak. Thus, the question arises as to whether PC/CHEMS liposomes will leak; i.e., is the CHEMS alone responsible for leakage of the liposomes? When the TPE is replaced by PC (7/3 PC/CHEMS) the release of contents at pH 4.5 is less than 1%/min (results not shown). Hence we can conclude that the PE head group is required for the rapid aggregation and destabilization.

**Effect of TPE/CHEMS Ratio and Liposome Concentration on pH-Induced Leakage.** The initial rate of leakage depends strongly on the molar fraction of TPE and on the concentration of liposomes, as can be seen in Figure 3. At equal lipid concentrations, the 7/3 TPE/CHEMS liposomes release their contents more rapidly and to a greater extent (Figure 3A) than do the 1/1 TPE/CHEMS liposomes (Figure 3B). This is consistent with the notion that TPE plays the dominant role in the leakage process. The initial rate of leakage increases with increasing liposome concentration, for both 7/3 and 1/1 TPE/CHEMS liposomes. The concentration dependence of leakage indicates that liposome aggregation is involved in the pH-induced leakage process. If leakage did not depend on liposome contact, then the curves would be lipid concentration independent.

Bentz et al. (1983a) have shown that if leakage of the liposomes requires aggregation and the leakage rate per liposome is rapid compared with the aggregation rate, then the graphs of leakage vs.  $X_0t$  (where  $X_0$  is the lipid concentration) for the different lipid concentrations will lie on the same line. For the 7/3 TPE/CHEMS liposomes (Figure 4A) it can be seen that at low liposome concentrations (1 and 2.5  $\mu$ M), the plots of leakage vs.  $X_0t$  do lie on the same curve. Therefore, at these low concentrations, leakage occurs only after the liposomes have aggregated, which implies that liposome contact is required to destabilize the bilayers. At the higher liposome concentrations, 5–50  $\mu$ M, leakage is significantly smaller for the same  $X_0t$  value. Therefore, at these high concentrations, the rate of leakage per liposome is no longer rapid compared with the rate of aggregation. For the 1/1 TPE/CHEMS liposomes (Figure 4B) it is evident that the rate of leakage is always slow compared to the rate of aggregation; i.e., if we could have looked at lower liposome concentrations (which was technically not possible), then we would have seen cographing of these curves. By way of comparison we also looked at the

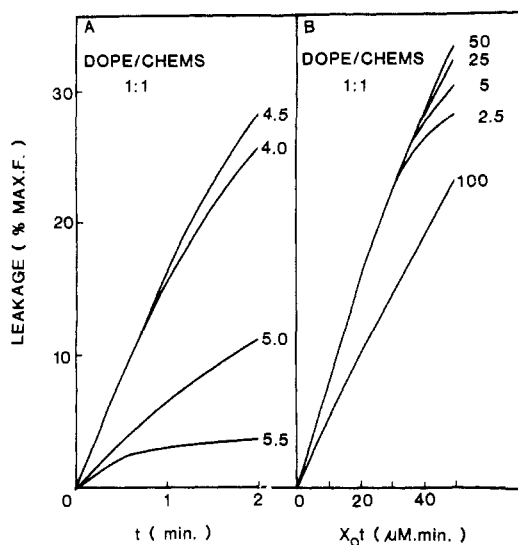


FIGURE 5: Effect of pH on DOPE/CHEMS, 1/1, liposomes and the concentration dependence of pH-induced leakage. 25  $\mu$ M DOPE/CHEMS, 1/1, liposomes were injected into buffers of different pHs, as indicated in the figure (50 mM sodium acetate/acetic acid, pH 5.5, 5.0, 4.5, and 4.0;  $\sim$ 150 mM NaCl to give the same osmotic strength as that of the encapsulated ANTS/DPX solution). Fluorescence was measured as a function of time (panel A). Relative leakage for various liposomal lipid concentrations in pH 4.5 buffer vs.  $X_0t$ , where  $X_0$  denotes the lipid concentration. The lipid concentrations (in  $\mu$ M) are indicated in the figure (panel B).

pH dependence and concentration dependence of leakage from 1/1 DOPE/CHEMS liposomes (Figure 5). The DOPE-containing liposomes release their contents in the same pH range as the TPE-containing liposomes, although they do so more rapidly and show some release at pH 5.5. The fact that the DOPE/CHEMS, 1/1, liposomes eventually release more contents at pH 4.5 than at pH 4.0 occurs well after the initial events ( $X_0t > 25 \mu$ M min) and so may be related to the leakage from larger aggregate structures. During the initial stage the release at the two pHs is identical (Figure 5A). It is clear that up to 50  $\mu$ M lipid concentration the leakage depends only on the value of  $X_0t$ ; i.e., the leakage occurs upon liposome contact and is rapid compared with the aggregation rate (Figure 5B).

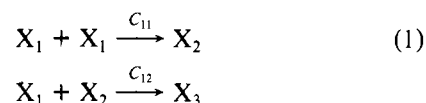
## Discussion

ANTS has been found to be a reliable and convenient fluorophore to continuously monitor pH-induced leakage. It satisfies the following minimum set of requirements. (1) Its fluorescence is relatively insensitive to pH above 4.0. At lower pH values, the fluorescence decreases dramatically, with an apparent  $pK_a$  of 3.5. The decrease in fluorescence is due to a protonation of the amine group and is associated with a blue shift in the excitation maximum. The emission maximum remains unchanged. (2) ANTS fluorescence can be efficiently quenched by DPX (Smolarsky et al., 1977; Figure 1). Unlike carboxyfluorescein and calcein, ANTS is not self-quenched at high concentrations, since there is no overlap between its excitation and emission spectra. The quenching of ANTS by DPX occurs by nonradiative energy transfer and depends on the average distance between the ANTS and DPX molecules. The rate of dequenching is therefore only dependent on the rate of dilution of ANTS and DPX into the medium, which is very rapid. The DPX concentration in the liposomes is chosen such that the liposomal fluorescence does not change the 0% leakage level up to  $\sim$ 50% leakage. Therefore, the leakage is measured directly by the relative change in fluorescence.<sup>2</sup> (3) ANTS diffuses only very slowly across intact liposomal membranes at or above pH 4.5. This is most

likely due to the sulfonic acid groups that remain charged at least down to pH 3.0 [compare, for example, with carboxyfluorescein: Szoka et al. (1979)]. Also, we have found that the initial leakage of ANTS and DPX from the PE/CHEMS liposomes occurs simultaneously. (4) When empty liposomes are incubated with 50 mM ANTS, 50 mM DPX, and 10 mM Tris, ANTS does not bind to the liposomes (see Materials and Methods), and therefore, like carboxyfluorescein and calcein, the ANTS/DPX system can be used to measure leakage from the aqueous space.

While it is difficult to accommodate neutral cholesterol esters into bilayers, a number of charged 3-*O*-cholesteryl esters can easily be incorporated into a liposome structure and some can even form liposomes by themselves (Colombat et al., 1981; Brockerhoff & Ramsammy, 1982). In this study, we have shown that it is possible to form stable liposomes from TPE or DOPE and the cholesteryl ester CHEMS at pH 7.5. When these liposomes are injected into pH 5.0–4.0 buffers, they aggregate and partially release their contents. The rate and extent of release increase with decreasing pH between 5.5 and 4.5 and it is most likely that protonation of the CHEMS initiates the destabilization. When PE is replaced by egg PC, the sensitivity to pH disappears. Since egg PC has the same acyl chain composition as TPE, it follows that the pH-induced destabilization requires the PE head group, and in fact, we have seen that the leakage increases with increasing molar fraction of TPE (Figure 3).

The initial rate of release of aqueous contents from the PE/CHEMS liposomes is strongly dependent on the liposome concentration, which shows that aggregation is involved in the process of leakage. It is possible to examine this question rigorously through an analysis of the kinetics of liposome aggregation (Bentz et al., 1983a,b). Briefly, the initial steps of liposome aggregation are



The aggregation of two liposomes  $X_1$  is governed by the kinetic rate constant for dimerization ( $C_{11}$ ). For the purposes of this analysis, we have ignored the dissociation reaction of the liposomes, although its effect on the leakage kinetics is discussed briefly below. The state of aggregation of a liposome suspension, i.e., the relative numbers of monomers, dimers, trimers, etc., depends only upon the product  $C_{11}X_0t$ , where  $X_0$  is the initial concentration of liposome monomers (or, equivalently, the lipid concentration) and  $t$  is the time (Bentz & Nir, 1981a,b).

Further analysis of this model has shown that when the plots of leakage vs.  $X_0t$  lie on the same curve regardless of the lipid concentration, then the liposomes remain stable until contact. Furthermore, the release of contents following contact must be rapid compared with the rate of aggregation (Bentz et al., 1983a). At low lipid concentrations, where the aggregation rates become sufficiently slow, the measured leakage will depend only on  $X_0t$ , since the rate of release of contents of each liposome following contact is independent of lipid concentration. At higher lipid concentrations when the aggregation is faster, the time required for the leakage from each liposome to evolve will make this process rate limiting. Hence, the higher lipid concentrations will show less leakage for a fixed value of  $X_0t$ . Of course, the lipid concentration at which the leakage rate per liposome becomes rate limiting to the overall process will depend upon the lipid composition and the pH. In the context of our results we will show that the liposomes leak very little, if at all, before contact. It is of interest to note

that the  $\text{Ca}^{2+}$ -induced leakage of carboxyfluorescein from PS liposomes has also shown the requirement of aggregation (Portis et al., 1979; Nir et al., 1980; Bentz et al., 1983a).

The case of DOPE/CHEMS, Figure 5B, is simplest in that the leakage depends only upon the state of aggregation for lipid concentrations up to 50  $\mu\text{M}$  and values of  $X_0t$  up to 40  $\mu\text{M}$  min, i.e., 16 min with 2.5  $\mu\text{M}$  lipid and 0.8 min for 50  $\mu\text{M}$  lipid. Although we are only interested in the initial events, it is of interest to note that at larger values of  $X_0t$  there is more leakage from 50  $\mu\text{M}$  liposomes than from the lower lipid concentrations. This result is expected if the aggregation is reversible, i.e., for low lipid concentrations at equilibrium there are relatively more monomers or liposomes that have not leaked (Bentz & Nir, 1981a). Once the lipid concentration reaches 100  $\mu\text{M}$  we see that the rate of liposome leakage becomes rate limiting since this curve lies below the others at all values of  $X_0t$ . For the 1/1 DOPE/CHEMS liposomes we can conclude also that there is no significant release of contents before bilayer contact.

For the case of 7/3 TPE/CHEMS, Figure 4A, we find that up to 2.5  $\mu\text{M}$  lipid the liposome leakage rate is rapid compared with the aggregation rate, as the data for 1  $\mu\text{M}$  lipid falls on the same curve when plotted against  $X_0t$ . The effect of aggregation reversibility also begins to emerge eventually. For this system the liposome leakage kinetics become rate limiting at 5  $\mu\text{M}$  lipid. We may conclude here that there is little or no leakage from the liposomes before contact. When the PE ratio is reduced to 1/1 TPE/CHEMS, Figure 4B, we find that the leakage is much reduced relative to the 7/3 TPE/CHEMS liposomes. Furthermore, we find that the rate of liposome leakage is always slow compared with the aggregation rate. Unfortunately, at lipid concentrations below 1  $\mu\text{M}$  the total fluorescence intensity was too small to be reliably measured.

In order to compare the behavior of these different liposome compositions we must consider the rate constants for aggregation, i.e., the values of  $C_{11}$  as defined in eq 1. The 1/1 TPE/CHEMS liposomes and the 1/1 DOPE/CHEMS liposomes differ only in the composition of the PE acyl chains; hence, their surfaces are essentially identical. While we cannot yet measure the aggregation rate constants, using the techniques applicable to other liposomal systems (Bentz & Nir, 1981b; Bentz et al., 1983a,b), we can assume that they are nominally equal for these two systems and depend mostly upon the extent of CHEMS protonation, i.e., the surface charge density, as is the case for other lipid systems. Since the overall leakage of the 1/1 DOPE/CHEMS liposomes is aggregation rate limited up to 50  $\mu\text{M}$  lipid, while the 1/1 TPE/CHEMS liposomes are not, at least down to 2.5  $\mu\text{M}$  lipid, we can deduce that the TPE produces a much slower leakage rate constant than does the DOPE. In other words, the 1/1 DOPE/CHEMS liposomes are more rapidly destabilized after contact than the 1/1 TPE/CHEMS liposomes. As discussed later this may reflect a greater propensity of DOPE to form nonbilayer structures at room temperature, e.g., the hexagonal  $\text{H}_{\text{II}}$  phase.

The 7/3 TPE/CHEMS liposomes become aggregation rate limiting below 2.5  $\mu\text{M}$  lipid, and at all lipid concentrations there is more leakage than with the 1/1 TPE/CHEMS and the 1/1 DOPE/CHEMS liposomes at the same extents of aggregation, i.e., values of  $X_0t$ . Assuming that the aggregation rate constants depend essentially on the surface charge density [i.e., greater surface charge density yields greater mutual electrostatic repulsion between the liposomes and a smaller aggregation rate constant (Bentz & Nir, 1981b)], then the 7/3 TPE/CHEMS liposomes should have a greater aggregation rate constant than the 1/1 TPE/CHEMS liposomes,

since there are more charged CHEMS groups in the latter case (Bentz, 1981). This would imply that the leakage rate per liposome for the 1/1 TPE/CHEMS liposomes is much smaller than that for the 7/3 TPE/CHEMS liposomes since in the latter case the aggregation rate constant is larger and the leakage is still aggregation rate limited up to 2.5  $\mu\text{M}$  lipid. Thus, the destabilization event is more rapid when there is more TPE in the bilayer.<sup>3</sup>

It is well-known that most types of PE, as well as some cardiolipins, will form nonbilayer structures under a wide variety of conditions, which include temperature and cation environment (Cullis & de Kruijff, 1979; Verkleij et al., 1980; Boggs et al., 1981; Harlos & Eibl, 1981; Bearer et al., 1982; Hope et al., 1983). However, the role of these nonbilayer structures in the initial events of bilayer destabilization has not been established. While TPE does not go to the  $\text{H}_{\text{II}}$  phase at room temperature and neutral pH (Mantsch et al., 1981), its behavior at low pH is not known. DOPE will undergo the  $\text{H}_{\text{II}}$  phase transition at room temperature (Cullis & de Kruijff, 1978), and this fact may underlie the more rapid leakage of the DOPE/CHEMS following contact at low pH (Figure 5). It is clear now that following the protonation of the CHEMS the liposome bilayer remains stable until it comes into contact with another bilayer. Thus, if the other morphologies are accessible to the TPE/CHEMS or DOPE/CHEMS (protonated) lipids, the transition to those morphologies begins with the contact of the two outer monolayers of the liposomes.

#### Acknowledgments

We thank Dr. D. Papahadjopoulos (Cancer Research Institute, UCSF) for the usage of the SLM 4000 fluorometer and members of the Drug Delivery Research Group for helpful discussions. The expert typing of Andrea Mazel and Rebekah Levy is appreciated.

**Registry No.** DOPE, 2462-63-7; CHEMS, 1510-21-0; ANTS, 117-42-0; DPX, 14208-10-7.

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<sup>3</sup> It turns out that when the leakage is aggregation rate limited, then the measured fluorescence depends only on the product  $\beta C_{11} X_0 t$ , where  $\beta$  is the fractional leakage per liposome per contact and  $0 < \beta < 1$  (Bentz et al., 1983a). A small aggregation rate with a large leakage per contact would yield the same measured leakage as a large aggregation rate with an appropriately smaller leakage per contact. Thus, we cannot yet say whether there is more leakage per contact with larger amounts of TPE in the bilayer.

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## Metastability in the Phase Behavior of Dimyristoylphosphatidylethanolamine Bilayers<sup>†</sup>

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**ABSTRACT:** A new subgel phase is demonstrated to occur in hydrated dimyristoylphosphatidylethanolamine (DMPE) by using dilatometric and calorimetric techniques. The formation of the subgel phase takes place very slowly at temperatures near 0 °C, but it can still be observed at 25 °C. Once formed, the subgel phase melts ( $\Delta H_h = 16.0 \pm 0.6$  kcal/mol and  $\Delta V = 0.085 \pm 0.014$  mL/g) directly into the liquid-crystalline phase at a temperature,  $T_h = 56.3$  °C, that is higher than the

gel to liquid-crystalline transition temperature,  $T_m = 49.6$  °C. Thus, the gel phase appears to be metastable over its entire temperature range. In this regard, DMPE behaves differently from dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine but similarly to dilaurylphosphatidylethanolamine. This unusual long-lived metastability provides cells an additional option in determining the properties of membranes.

**L**ow-temperature phases in phospholipid bilayers and the phase transitions into such phases are important references with which to compare the biologically relevant high-temperature phase,  $L_\alpha$ , and from which to obtain quantitative information concerning the molecular interactions that determine bilayer structure (Nagle & Wilkinson, 1978). The

discovery by Chen and co-workers (Chen et al., 1980) of the subtransition in saturated phosphatidylcholines having 16, 17, or 18 carbons per chain has been followed by some detailed studies of the kinetics of formation and the density of the new subgel phase (Nagle & Wilkinson, 1982) and by structural studies of the subgel phase using diffraction and spectroscopy (Fuldner, 1981; Ruocco & Shipley, 1982; Cameron & Mantsch, 1982). Characterization of the subgel phase promises to provide an even simpler reference state than either the  $L_\beta$  or the  $P_\beta$  phase.

It is of interest, therefore, to determine if subgel phases occur in other lipids as well as in the phosphatidylcholines. In an

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