H^+- and Ca^{2+}-Induced Fusion and Destabilization of Liposomes†

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ABSTRACT: A new liposome fusion assay has been developed that monitors the mixing of aqueous contents at neutral and low pH. With this assay we have investigated the ability of H^+ to induce membrane destabilization and fusion. The assay involves the fluorophore 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and its quencher *N,N'-p*-xylylenebis(pyridinium bromide) (DPX). ANTS is encapsulated in one population of liposomes and DPX in another, and fusion results in the quenching of ANTS fluorescence. The results obtained with the ANTS/DPX assay at neutral pH give kinetics for the Ca^{2+}-induced fusion of phosphatidylserine large unilamellar vesicles (PS LUV) that are very similar to those obtained with the Tb^{3+}/dipicolinic acid (DPA) assay [Wilschut, J., & Papahadjopoulos, D. (1979) *Nature (London) 281*, 690–692]. ANTS fluorescence is relatively insensitive to pH between 7.5 and 4.0. Below pH 4.0 the assay can be used semiquantitatively by correcting for quenching of ANTS due to protonation. For PS LUV it was found that, at pH 2.0, H^+ by itself causes mixing of aqueous contents, which makes H^+ unique among the monovalent cations. We have shown previously that H^+ causes a contact-induced leakage from liposomes composed of phosphatidylethanolamine and the charged cholesteryl ester cholesteryl hemisuccinate (CHEMS) at pH 5.0 or below, where CHEMS becomes protonated. Here we show that H^+ causes lipid mixing in this pH range but not mixing of aqueous contents. This result affirms the necessity of using both aqueous space and lipid bilayer assays to comprehend the fusion event between two liposomes.

The interaction of divalent and monovalent cations with liposomes composed of acidic lipids has been intensively studied (Nir et al., 1983a). Typically, the results are that divalent cations induce the fusion of these liposomes, while the monovalent cations induce only aggregation without fusion. The exception to this rule is H^+, which induces aggregation and lipid mixing of phosphatidylserine (PS)[1] SUV (Papahadjopoulos et al., 1977; Ohki et al., 1982), phosphatidylethanolamine (PE) liposomes (Kolber & Haynes, 1979; Pryor et al., 1983), PS/PE SUV (Hope et al., 1983), PE/oleic acid multilamellar vesicles (MLV) (Düzgüneş et al., 1983), and PE/palmitoylhomocysteine SUV (Connor et al., 1984). While the conclusion that H^+ can destabilize PS and PE bilayers remains, the crucial question is still unanswered: does H^+ induce liposome fusion with the concomitant mixing of bilayers and encapsulated contents, like Ca^{2+}, or does it cause lysis and collapse of the liposome structure accompanied by lipid mixing? We have addressed this question here, and the answer is that H^+ can do either, depending upon the lipids involved.

The study of membrane fusion, using liposomes as model systems, was greatly accelerated by the development of fluorescence assays competent to continuously monitor the mixing of the encapsulated aqueous contents (Ingolia & Koshland, 1978; Holz & Stratford, 1979; Wilschut & Papahadjopoulos, 1979; Hoekstra et al., 1979) and the merging of bilayers (Keller et al., 1977; Gibson & Loew, 1979; Owen, 1980; Vanderwerf & Ullman, 1980; Uster & Deamer, 1981; Struck et al., 1981). When liposome fusion is defined as the concomitant mixing of bilayers and aqueous contents, which we believe is the biologically relevant definition, then it is clear that the two types of assays are complementary. The lipid mixing assays cannot discriminate between this fusion and the case where the liposomes lyse first and the bilayers mix later. Furthermore, care must be exercised to exclude the case where the lipid probe merely exchanges between the liposomes, either by diffusion through the medium or by exchange between apposed bilayers, without the merging of the bilayers.

In general, we can conceive of three possible interactions between the liposomes: fusion, i.e., mixing of aqueous contents and bilayer components; lysis, i.e., no mixing of aqueous contents but leakage and mixing of bilayer components; and mixing of bilayer components with neither leakage nor mixing of aqueous contents. This last case may be due to either outer monolayer mixing, while the inner monolayers remain intact, or probe exchange between the apposed bilayers without the concomitant flow of unlabeled lipid. In this study we show examples of all three cases.

The most rigorously studied aqueous space fusion assay is the terbium/dipicolinate (Tb^{3+}/DPA) assay, which measures the mixing of liposome contents via the enhancement of Tb^{3+} fluorescence following chelation by DPA (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980, 1981; Nir et al., 1980; Bentz et al., 1983a,b). At or near neutral pH, this assay has been used to examine the cation-induced aggregation and fusion of liposomes containing acidic phospholipids (Nir et al., 1983a,b; Bentz et al., 1983a,b, 1985a; Schuber et al., 1983). Unfortunately, below pH 5.0 the DPA becomes protonated

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[1] Abbreviations: PE, phosphatidylethanolamine; PS, phosphatidylserine; TPE, phosphatidylethanolamine prepared from egg phosphatidylcholine by transesterification; 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, *N,N'-p*-xylylenebis(pyridinium bromide); DPA, dipicolinic acid; His, L-histidine; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; EDTA, ethylenediaminetetraacetic acid.

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and the $Tb(DPA)_3^{3-}$ fluorescence falls off rapidly (Barela & Sherry, 1976), presumably due to the fact that the DPA is now incapable of chelating with Tb^{3+}.

In order to study the effect of low pH on liposome stability, we developed a new aqueous space fusion assay. The assay is based on the quenching of 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS) fluorescence by *N,N'-p*-xylylenebis(pyridinium bromide) (DPX) (Smolarsky et al., 1977; Ellens et al., 1984). ANTS is encapsulated in one population of liposomes and DPX in the other. Mixing of aqueous contents inside of the liposomes results in quenching of ANTS fluorescence. Dilution of DPX in the medium prevents quenching of ANTS fluorescence outside the liposomes. ANTS fluorescence is relatively independent of pH between 4.0 and 7.5 (Ellens et al., 1984). Below pH 4.0 ANTS is quenched due to protonation of the amine group. However, the assay can still be used semiquantitatively to measure mixing of aqueous contents below pH 4.0 (down to pH 2.0) by correcting for fluorescence quenching due to protonation. The assay can be used to directly measure leakage of contents down to pH 3.0.

With this assay it is observed that H^+ by itself at pH 2.0 induces fusion and mixing of aqueous contents of PS LUV. This makes H^+ unique among the monovalent cations in being able to induce the fusion of these liposomes (Bentz et al., 1983b; Nir et al., 1983b).

Previously, it has been demonstrated that H^+ causes a contact-induced leakage from liposomes composed of phosphatidylethanolamine and cholesteryl hemisuccinate (CHEMS) at pH values of 5.0 or below (Ellens et al., 1984). Here we show that there is H^+-induced mixing of lipids in the same pH range, using resonance energy transfer of lipid probes (Rh-PE and NBD-PE; Struck et al., 1981). Although there is lipid mixing and contact-induced leakage of PE/CHEMS liposomes at or below pH 5.0, H^+ does not cause mixing of aqueous contents. This is the first membrane system where lipid mixing and leakage occur without the actual fusion of the liposomes.

Materials and Methods

Phosphatidylserine (PS) from bovine brain, 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). Cholesteryl hemisuccinate (CHEMS) was from Sigma. $TbCl_3$ was obtained from Alfa (Danvers, MA); dipicolinic acid (DPA, 2,6-pyridinedicarboxylic acid), Tes [*N*-tris[(hydroxymethyl)methyl]-2-aminoethanesulfonic acid], L-histidine, and Tris-HCl [tris(hydroxymethyl)aminomethane hydrochloride] were from Sigma. 1-Aminonaphthalene-3,6,8-trisulfonic acid (disodium salt) (ANTS) and *N,N'-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.

Large unilamellar liposomes (LUV) were prepared according to Szoka & Papahadjopoulos (1978). For the ANTS/DPX fusion assay the liposomes contained either (i) 25 mM ANTS and 40 mM NaCl, (ii) 90 mM DPX, or (iii) 12.5 mM ANTS, 45 mM DPX, and 20 mM NaCl. All solutions were buffered with 10 mM Tris-HCl at pH 7.5. For the Tb^{3+}/DPA fusion assay the liposomes contained either (i) 2.5 mM $TbCl_3$ and 50 mM sodium citrate, (ii) 50 mM DPA and 20 mM NaCl, or (iii) 1.25 mM $TbCl_3$, 25 mM DPA, 25 mM sodium citrate, and 10 mM NaCl. All solutions were buffered with 2 mM L-histidine and 2 mM Tes, pH 7.4. In all cases the encapsulated solutions were isosmotic to the buffers used for the column chromatography and in the fusion experiments. The liposomes were extruded successively through polycarbonate membranes (Bio-Rad) with 0.2- and 0.1-μm pores (Olson et al., 1979). The liposomes were separated from unencapsulated material on Sephadex G-75 (Pharmacia) by using 100 mM NaCl, 2 mM Tes, 2 mM L-histidine (pH 7.4), and 1 mM EDTA as the elution buffer. Liposomal lipid concentrations were determined by phosphate analysis (Bartlett, 1959). With PS the encapsulated volume was 4.9 μL/μmol of lipid for ANTS and ANTS/DPX liposomes and 5.3 μL/μmol of lipid for Tb^{3+} and Tb^{3+}/DPA liposomes. With TPE/CHEMS (7/3) the encapsulated volume was 1.9 μL/μmol of total lipid for ANTS and ANTS/DPX liposomes.

We determined that there was no significant binding of ANTS to the liposomes by incubating "empty" PS liposomes with 12.5 mM ANTS 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 $\leq$1% of the total fluorescence that the liposomes would have encapsulated. ANTS is therefore a true marker of the aqueous space of the liposomes.

DPX is highly water soluble and, when coencapsulated with ANTS, efficiently quenches ANTS fluorescence by collisional transfer. DPX is therefore presumably encapsulated in the aqueous compartment of the liposomes. In addition, both ANTS and DPX remain trapped for extended periods of time. DPX is an organic salt molecule, and it can conceivably bind to PS. DPX binding cannot be assayed directly. We therefore used the Ca^{2+}-induced fusion of PS liposomes, monitored with the Tb^{3+}/DPA assay, to determine if there is significant binding of DPX to PS, since Ca^{2+}-induced fusion of PS liposomes is extremely sensitive to binding of other divalent cations (Wilschut et al., 1981; Bentz & Düzgüneş, 1985). Even at 5 mM DPX there is no effect on the initial rates of Ca^{2+}-induced aggregation and fusion of PS, and we therefore conclude that *if* there is binding of DPX to the PS molecules, the binding is very weak. Most, if not all, DPX will therefore be removed from the outside of the liposomes after dilution on the Sephadex column. In addition, it should be noted that PS liposomes can be made in 150 mM DPX (the highest concentration tested) whereas they cannot be made even in 2 mM Ca^{2+}, due to precipitation of the Ca^{2+}/PS complex. Although we cannot exclude an interaction between DPX and PS, if it occurs it does not interfere with the ANTS/DPX fusion assay.

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. The Tb^{3+}/DPA fusion assay was done as described by Wilschut et al. (1980) and Bentz et al. (1983b, 1985a). Excitation was at 276 nm. Emission was measured through a Corning 3-68 cutoff filter (>530 nm).

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. The fluorescence scale is calibrated with the fluorescence of a 1/1 (or a 1/9) mixture of ANTS and DPX liposomes in NaCl buffer (100 mM NaCl, 2 mM His,

2 mM Tes, and 0.1 mM EDTA, pH 7.4) 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. Leakage is measured with liposomes containing both ANTS and DPX. 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 lysed liposomes (using Triton X-100) is set to 100% leakage. Excitation was at 384 nm and emission at >530 nm. On the figures, fusion (% max Q) is equal to 100 minus the recorded fluorescence, which is equal to the percentage of ANTS that is quenched by DPX at that time.

Lipid mixing was monitored as described by Struck et al. (1981). Liposomes composed of TPE and CHEMS in a 7/3 molar ratio, containing 1 mol % each of the fluorescent lipid analogues NBD-PE and Rh-PE, were mixed with TPE/CHEMS (7/3) 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 a decreased energy transfer between NBD-PE and Rh-PE as the two probes are diluted. The 100% fluorescence level was set with TPE/CHEMS (7/3) liposomes containing 0.1 mol % each of NBD-PE and Rh-PE at the same total lipid concentration as was used in the lipid mixing experiment. This was done because Triton X-100 severely quenches NBD fluorescence (Tanaka & Schroit, 1983). In the range of pH 7.4–4.0 there is only a small effect of H^+ on the fluorescence intensity of NBD, <10%; hence, the 100% fluorescence level in the H^+-induced lipid mixing experiments was set at pH 7.4. When the liposomes containing 0.1 mol % of the fluorophores were injected into a pH 4.5 buffer, there was no change in the fluorescence intensity. 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 25 °C. 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 NaCl buffer (100 mM NaCl, 2 mM His, 2 mM Tes, and 0.1 mM EDTA, pH 7.4). 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 ~50 mM NaCl, pH 2.0–5.5). In *all* cases, the buffers were made isosmotic to the encapsulated contents of the liposomes by using NaCl, and the osmolality was measured by using a vapor pressure osmometer (Wescor, Inc., Logan, UT). The results were recorded on an Omniscribe chart recorder at high chart speeds when necessary.

RESULTS

ANTS/DPX Fusion Assay. The ANTS/DPX fusion assay is based on the quenching of ANTS fluorescence by DPX. ANTS is encapsulated in one population of liposomes and DPX in another, and the mixing of aqueous contents is registered as a decrease in fluorescence intensity. ANTS and DPX meet the requirements for a competent fusion assay: (i) They are both encapsulated in the aqueous space of the liposomes and their rate of leakage from intact liposomes is negligible (see Materials and Methods). (ii) The quenching reaction is fast, allowing for the measurement of fast fusion kinetics. (iii) Since ANTS in the liposomes is not self-quenched, mere leakage of ANTS will not result in a change of fluorescence intensity (Ellens et al., 1984) and dilution of

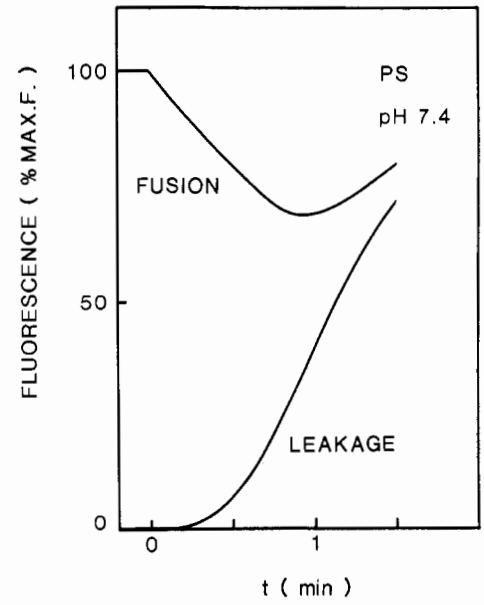

FIGURE 1: Ca^{2+}-induced fusion and leakage of PS liposomes monitored with the ANTS/DPX fusion assay at neutral pH. 25 μM ANTS liposomes and 25 μM DPX liposomes or 50 μM ANTS/DPX liposomes were suspended in 1 mL of NaCl buffer containing 0.1 mM EDTA. 5 mM Ca^{2+} was added at $t = 0$. Fusion of the ANTS and the DPX liposomes and leakage from the ANTS/DPX liposomes are measured as described under Materials and Methods.

DPX in the medium prevents quenching of ANTS fluorescence outside the liposomes. Quenching of ANTS fluorescence will therefore only occur upon mixing of aqueous contents inside of the liposomes.

Throughout this paper we have quantified fusion as the percentage of ANTS that is quenched by DPX at a given time. The same definition has been used for the Tb^{3+}/DPA assay both here and previously (Wilschut et al., 1980, 1981; Nir et al., 1980, 1982; Bentz et al., 1983a,b, 1985a). This percentage must not be confused with the percentage of liposomes that have fused or undergone lipid mixing. The number of fused liposomes increases with time; however, the percentage of ANTS quenched by DPX at a given time depends upon both the fusion kinetics of the liposomes and the leakage kinetics of the fused products. We have shown previously for aqueous contents mixing assays that one can rigorously combine the fusion signal with a fraction of the leakage signal to obtain the total destabilization, i.e., what the fusion signal would be if there were no leakage from the fused structures (Nir et al., 1980; Bentz et al., 1983a, 1985a). This corrected fluorescence is what can be compared with the lipid mixing assay. From this corrected signal one can obtain the rate constants of aggregation and destabilization. Here, however, we are primarily concerned with the behavior of the ANTS/DPX assay and especially how this assay behaves at low pH where the Tb^{3+}/DPA assay cannot be used.

We investigated the mixing of aqueous contents of PS LUV during Ca^{2+}-induced fusion using the ANTS/DPX fusion assay (Figure 1). The fluorescence intensity decreases sharply immediately after the fusion reaction is started by the addition of Ca^{2+}. With 5 mM Ca^{2+} the fluorescence quenching reaches a maximum in about 1 min. Thereafter, the fluorescence intensity increases again. The increase is caused by a release of the contents of the fused liposomes into the medium. This release is studied directly in a separate experiment using liposomes containing both ANTS and DPX. The increase in fluorescence that occurs when ANTS and DPX are released from the liposomes is monitored under conditions identical with

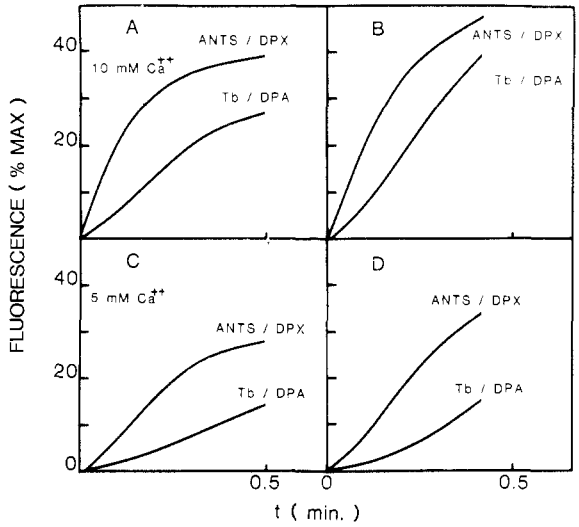

FIGURE 2: Comparison of the ANTS/DPX and Tb^{3+}/DPA fusion assays using the Ca^{2+}-induced fusion of PS LUV. A 50 μM 1:1 mixture of ANTS and DPX liposomes or a 50 μM 1:1 mixture of Tb^{3+} and DPA liposomes was suspended in 1 mL of NaCl buffer containing 0.1 mM EDTA. Ca^{2+} was added at $t = 0$. Panels A and C show the fusion signals for both assays with 10 and 5 mM Ca^{2+}, respectively. Panels B and D show the corrected fusion signals for both assays, obtained by adding half of the leakage signal (not shown) of ANTS/DPX liposomes to the fusion signal for the ANTS and DPX liposomes or by adding half of the dissociation signal (not shown) of the Tb^{3+}/DPA liposomes to the fusion signal for the Tb^{3+} and DPA liposomes (Bentz et al., 1983a, 1985a). Note that all fusion curves using the ANTS/DPX assay are given in units of percent maximum quenching, i.e., 100 minus the measured fluorescence curve.

those in the fusion experiment. The Ca^{2+}-induced leakage of ANTS and DPX from the PS liposomes is clearly delayed compared to the mixing of contents (Figure 1). This indicates that at least during the initial fusion events leakage is very low. The massive release that occurs as a secondary event to fusion is most likely due to a collapse of the fused liposomes (Wilschut et al., 1980; Bentz et al., 1985a). It is also clear from these data that Ca^{2+} has no effect on ANTS fluorescence.

In Figure 2, we compare the ANTS/DPX and Tb^{3+}/DPA fusion assays using the Ca^{2+}-induced fusion of PS LUV. In panel A, the fusion signal for 10 mM Ca^{2+} is shown. The ANTS/DPX signal is affected by leakage of contents, whereas the Tb^{3+}/DPA signal is affected by leakage and the influx of medium within the fused structures, since the Tb^{3+}/DPA complex is disrupted by both Ca^{2+} and 0.1 mM EDTA in the medium (Bentz et al., 1983b, 1985a). Thus, we have shown in panel B the corrected fusion signal for both assays, i.e., the signal that would be obtained if there were no leakage of contents or influx of medium during fusion of the liposomes. The method of this correction has been described in detail (Nir et al., 1980, 1983a; Bentz et al., 1983a,b, 1985a), and a brief description is given in the figure legend. Panel C shows the fusion signal for 5 mM Ca^{2+}, and panel D shows the corrected curves. Since the leakage of contents is small initially, the uncorrected curves yield qualitatively the same information as the corrected curves; however, since the assays respond differently to leakage, the corrected curves are more relevant in the comparison.

It is clear that the two assays do not give precisely the same results. The ANTS/DPX assay monitors a faster fusion event than the Tb^{3+}/DPA assay. Qualitatively, the two assays agree: the threshold Ca^{2+} concentration needed to produce fusion is the same ($\sim$2 mM); neither assay shows mixing of contents in 10 mM Mg^{2+} (data not shown); there is a proportionate increase in the kinetics as the Ca^{2+} concentration is raised from

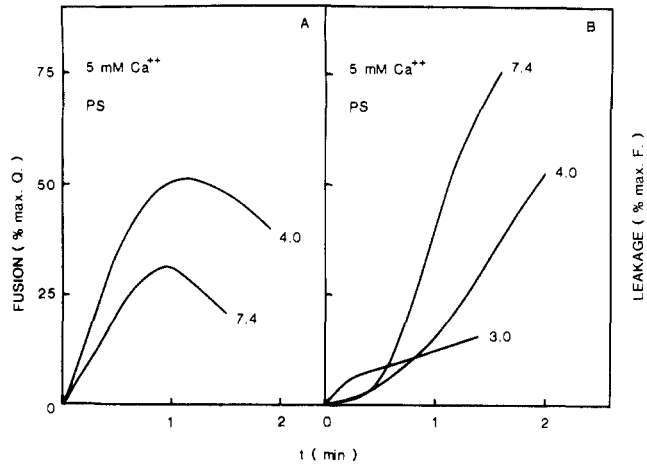

FIGURE 3: Effect of H^+ on Ca^{2+}-induced fusion and leakage of PS liposomes. (A) 25 μM ANTS liposomes and 25 μM DPX liposomes were injected into buffers of pH 7.4 and 4.0 (as indicated in the figure) containing 5 mM Ca^{2+}. Fusion was measured as described under Materials and Methods and in Figure 2. (B) 50 μM ANTS/DPX liposomes were injected into buffers of pH 7.4, 4.0, and 3.0 (as indicated in the figure), and leakage was measured as described under Materials and Methods.

3 (not shown) to 10 mM Ca^{2+} and as the lipid concentrations range from 1 to 62.5 μM (data not shown). At present, we do not know what factors underlie the quantitative differences between the two assays. However, we have recently shown with the Tb^{3+}/DPA assay that the method of preparation of PS LUV, extruded through 0.1-μm filters, can affect the observed kinetics (Bentz et al., 1985a). Specifically, small changes in the average size of the liposomes, as measured by dynamic light scattering, led to significant changes in the fusion kinetics; i.e., a smaller average size gave faster kinetics. A more detailed knowledge of the size distributions of the liposomes, as prepared for the Tb^{3+}/DPA and the ANTS/DPX assays, will be needed to understand whether the quantitative difference between the two assays is due only to shifts in the size distributions. It is important to point out that when compared with previously published results for other aqueous contents mixing assays (Hoekstra et al., 1979; Kendall & McDonald, 1982), the ANTS/DPX assay is almost identical with the Tb^{3+}/DPA assay.

ANTS/DPX Assay at Low pH. The ANTS/DPX fusion assay can be used directly between pH 7.4 and 4.0 since ANTS fluorescence is essentially independent of pH in this range (Ellens et al., 1984). Below pH 4.0, in addition to the quenching by DPX in the fused liposomes, ANTS is also partly quenched by protons, and therefore the assay cannot be used to quantitate fusion directly. However, as we will show below, it can be used to demonstrate fusion in a semiquantitative manner. Leakage on the other hand is measured as an *increase* in fluorescence, due to the relief of quenching upon dilution of DPX in the medium, and can therefore be measured directly down to at least pH 3.0.

The Ca^{2+}-induced fusion and leakage of PS liposomes was measured at various pH values in the presence of 5 mM Ca^{2+} (Figure 3). At pH 4.0 the Ca^{2+}-induced leakage is inhibited, the maximum fusion signal with 5 mM Ca^{2+} is increased, and the overall fusion reaction is faster. It should be noted that the initial Ca^{2+}-induced fusion of PS LUV at pH 7.4 is essentially nonleaky, and the massive leakage that occurs after the first 20 s (Figure 3B) is due to a collapse of the fused liposomes into anhydrous cochleate structures (Papahadjopoulos et al., 1975; Portis et al., 1979; Wilschut et al., 1980). It is this collapse into cochleates that is inhibited at pH 4.0,

Table I: Retention of Contents after Separation of Released ANTS from PS Liposomes[a]

				intraliposomal			
	in void vol (%)	in elution vol (%)	leakage from chart (%)	quenching (%)	pH	quenching due to H^+ (%)	quenching due to DPX (%)
ANTS liposomes	67	32	26	15	~4.0	15	0
mixture of ANTS and DPX liposomes	62	35	26	38	~4.0	15	23

[a] 50 μM ANTS liposomes or a mixture of 25 μM ANTS and 25 μM DPX liposomes is injected into a pH 2.0 buffer not containing any Ca^{2+}. Ten seconds after injection of the liposomes the pH of the medium is neutralized by the injection of excess NaOH, and the incubations are chromatographed over Sephadex G-75. The fluorescence remaining with the liposomes eluting in the void volume is measured without and with Triton X-100, and this gives the intraliposomal quenching. The ANTS that has leaked from the liposomes appears in the elution volume. "Leakage from chart" is given by the increase in fluorescence following addition of NaOH as described in the legend to Figure 4.

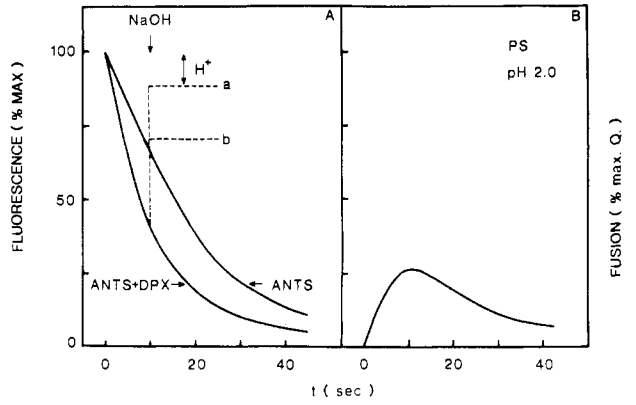

FIGURE 4: H^+-induced fusion of PS liposomes. (A) 50 μM ANTS liposomes or a mixture of 25 μM ANTS and 25 μM DPX liposomes is injected into a pH 2.0 buffer not containing any Ca^{2+}, and the rate of quenching of ANTS fluorescence in both cases is measured as described under Materials and Methods. In some cases the pH of the medium is neutralized 10 s after injection of the liposomes by the injection of excess NaOH (dashed lines). The *upper* dashed line (a) shows the dequenching for just the ANTS-containing liposomes. The distance between this level and 100% gives the quenching of ANTS inside of the liposomes due to protonation, denoted H^+. The dequenching is due to ANTS that has leaked from the liposomes during fusion and that is restored to neutral pH after NaOH addition. The *lower* dashed line (b) shows the dequenching for the ANTS- and DPX-containing liposomes. The distance between the lower curve and this dashed line is due to ANTS that has leaked during fusion and has been restored to neutral pH. This is referred to as "leakage from chart" in Table I. (B) The difference between the two solid curves in panel A gives the quenching of ANTS by DPX.[2]

and the inhibition of the collapse largely accounts for the higher fusion signal at this pH. That the initial fusion kinetics are more rapid at pH 4.0 can be accounted for by the aggregation step. With 50 μM PS liposomes and 5 mM Ca^{2+} the fusion at neutral pH is essentially aggregation rate limited (Nir et al., 1982; Bentz et al., 1983a), and the faster overall fusion reaction at pH 4.0 is therefore due to an increase in the aggregation rate rather than the fusion rate per se, as is seen with increasing concentrations of other monovalent cations as well (Bentz et al., 1983b). Without Ca^{2+} there is no leakage or fusion of PS liposomes at pH 4.0. At pH 3.0 with 5 mM Ca^{2+} there is an initial burst of leakage, but the collapse into cochleates is inhibited relative to pH 4.0 or 7.4. The initial burst of leakage indicates that under these conditions H^+ is directly involved in the membrane destabilization. The liposomes do not leak at pH 3.0 without Ca^{2+}, and this is ascribed to the lack of aggregation.

H^+-Induced Fusion of PS Liposomes. In a fusion experiment below pH 4.0, quenching of ANTS fluorescence can be brought about by DPX following mixing of contents of ANTS and DPX liposomes and by protons that have entered the liposomes. The ANTS/DPX fusion assay can be used semiquantitatively below pH 4.0 by correcting the quenching caused by both DPX and H^+ (in a mixture of ANTS and DPX liposomes) for the quenching by H^+ alone (with just ANTS liposomes). The difference between the two curves gives the quenching caused by DPX alone. However, the subtraction of the two curves does not account for the fact that the maximum fluorescence or "100%" level is decreasing in time also. Thus, the fusion curve in Figure 4B underestimates the true extent of aqueous contents mixing.[2]

Upon injection of ANTS-containing PS liposomes into a pH 2.0 buffer there is a rapid quenching of ANTS fluorescence (Figure 4A). Quenching is faster however upon injection of a 1/1 mixture of ANTS and DPX liposomes (Figure 4A), indicating that there is an H^+-induced mixing of aqueous contents of the ANTS- and DPX-containing liposomes. To verify this, we stopped the pH-induced fusion process after 10 s by adding excess NaOH, reisolated the liposomes from the medium, and found that ANTS and DPX were contained in the same fused liposomes. Furthermore, when the fusion reaction was stopped, the fluorescence signal increased instantaneously and then remained at a constant level as shown

[2] The nature of this underestimate, and its correction, can be best explained by an example. Suppose that all of the liposomes fused once to form doublets and there is no leakage of contents. Since we begin here with equal concentrations of ANTS and DPX liposomes, half of the encapsulated ANTS will be within doublets that contain DPX, and the other half of the ANTS will be in doublets that contain no DPX. The ANTS in the DPX-containing doublets is essentially completely quenched, and so these doublets contribute no fluorescence. The measured fluorescence will emanate only from the doublets formed from the fusion of two ANTS liposomes, i.e., AA doublets. If the intraliposomal pH is 4.0 or above, then the ANTS in these doublets is fully fluorescent and we would see 50% maximal fluorescence, since the 100% level is set to the fluorescence intensity of all of the encapsulated ANTS at pH 7.4. For the experiment with just the ANTS liposomes, the fluorescence would remain at 100%; hence, the difference between the two curves is 100% - 50% = 50%. Thus, the percent of maximal fluorescence equals the amount of ANTS that is quenched by DPX. However, if the intraliposomal pH is below 4.0, then the measured fluorescence would be 50% times the relative ANTS fluorescence at that pH. For example, at pH 3.0 the protonation of the ANTS reduces its fluorescence to 32% maximal fluorescence [see Figure 1 of Ellens et al. (1984)]. If the intraliposomal pH were 3.0, then the measured fluorescence of pure ANTS liposomes would show 32% maximal fluorescence while the mixture of the ANTS + DPX liposomes would show 50% × 0.32 = 16% maximal fluorescence. Thus, the difference between the two curves, 32% - 16% = 16%, considerably underestimates the amount of ANTS that is quenched by DPX; i.e., the extent of fusion is underestimated. The acetate buffer rapidly transports protons into the liposomes in the form of acetic acid, so the intraliposomal pH will rapidly fall, and the bilayer destabilization during fusion will accelerate this decrease. Since the fluorescence curve obtained for the pure ANTS liposomes is due to both leakage of ANTS into the medium and the passage of protons into the fusing liposomes, it is not yet possible to accurately measure the intraliposomal pH. If the intraliposomal pH were known, then the measured fluorescence could be divided by the relative ANTS fluorescence at that pH to obtain the percentage of ANTS not in contact with the DPX, e.g., 16%/0.32 = 50% for the latter example. In any event, the curve shown in Figure 4B shows substantial mixing of aqueous contents. Although this is an underestimate of the true extent of content mixing, it still proves that H^+ will induce the fusion of PS LUV.

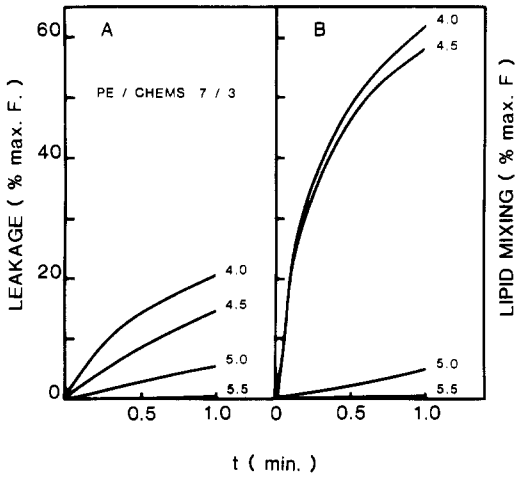

FIGURE 5: Effect of H^+ on TPE/CHEMS (7/3) liposomes. (A) 50 μM ANTS/DPX liposomes were injected into buffers of pH 4.0, 4.5, 5.0, and 5.5 (as indicated in the figure), and leakage was measured as described under Materials and Methods. (B) A 50 μM 1:9 mixture of the fluorescent NBD-PE/Rh-PE/TPE/CHEMS (0.1/0.1/7/3) liposomes and the blank TPE/CHEMS (7/3) liposomes was injected into buffers of pH 4.0, 4.5, 5.0, and 5.5 as indicated in the figure, and lipid mixing was measured as described under Materials and Methods.

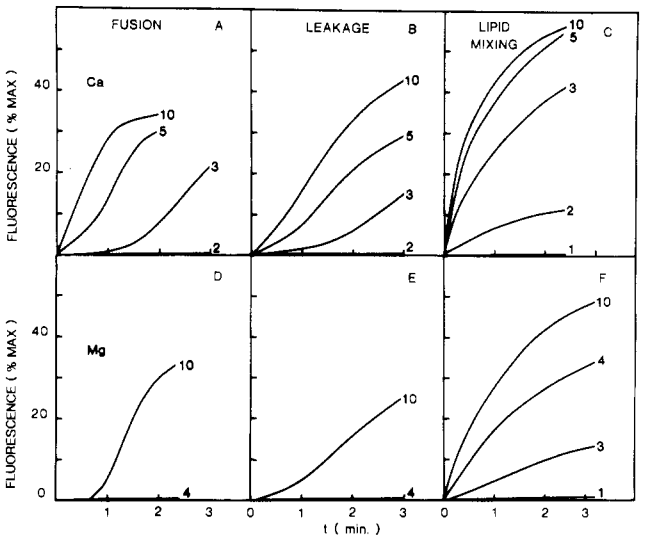

FIGURE 6: Ca^{2+}- and Mg^{2+}-induced fusion, leakage and lipid mixing of TPE/CHEMS (7/3) liposomes. (A and D) 5 μM ANTS and 45 μM DPX liposomes were suspended in 1 mL of NaCl buffer containing 0.1 mM EDTA. Different concentrations of Ca^{2+} (2, 3, 5, and 10 mM, as indicated in panel A) or Mg^{2+} (4 and 10 mM, as indicated in panel D) were added at $t = 0$. (B and E) 50 μM ANTS/DPX liposomes were suspended in 1 mL of NaCl buffer containing 0.1 mM EDTA. Different concentrations of Ca^{2+} (2, 3, 5, and 10 mM, as indicated in panel B) and Mg^{2+} (4 and 10 mM, as indicated in panel E) were added at $t = 0$. (C and F) A 50 μM 1:9 mixture of the fluorescent NBD-PE/Rh-PE/TPE/CHEMS (0.1/0.1/7/3) and the blank TPE/CHEMS (7/3) liposomes was suspended into 1 mL of NaCl buffer containing 0.1 mM EDTA. At $t = 0$ different concentrations of Ca^{2+} (1, 2, 3, 5, and 10 mM, as indicated in panel C) or Mg^{2+} (1, 3, 4, and 10 mM, as indicated in panel F) were added. Fusion, leakage, and lipid mixing were measured as described under Materials and Methods. Note that the fusion curves using the ANTS/DPX assay are given in units of percent maximum quenching, i.e., 100 minus the measured percent maximum fluorescence signal.

in Figure 4A (dashed lines). Quenching in the medium is abolished due to the increased pH. This means that the increased quenching with the ANTS/DPX mixture actually reflects an increased quenching of ANTS in the liposomes. It should be noted that the increase in fluorescence measures the leakage. Since the increase in fluorescence is the same for both the mixture of ANTS and DPX liposomes and for just the ANTS liposomes, this shows that the leakage is the same for both cases.

The incubations that were stopped after 10 s were chromatographed on Sephadex G-75, and the fluorescence in the void volume and elution volume was measured (Table I). The liposomes eluting in the void volume were measured without and with Triton X-100 and it is shown that in the ANTS and DPX mixture there is a much greater quenching of fluorescence than with the ANTS liposomes alone, indicating that ANTS and DPX are contained within the same liposome. Differences in intraliposomal quenching could conceivably be due to differences in intraliposomal pH. We therefore estimated the intraliposomal pH by determining the fluorescence in the liposomes at different wavelengths and comparing this to an ANTS standard curve obtained at different pHs. The pH in the liposomes in both incubations was around 4.0. This rules out the possibility that the 38% quenching in the ANTS and DPX mixture is due to protons alone and is compatible with the small amount of quenching (15%) in the ANTS liposomes. The additional quenching in the ANTS and DPX mixture therefore has to be due to ANTS and DPX contained within the same fused liposomes. The fluorescence increase after NaOH injection (Figure 4 and Table I) and the amount of fluorescence in the elution volume of the column (Table I) are two measures of the H^+-induced leakage, which are in reasonably good agreement. It should be noted that the H^+-induced fusion seems to be more leaky than the Ca^{2+}-induced fusion at pH 7.4 (compare with Figure 3).

Effect of H^+ on Liposomes Composed of TPE and CHEMS. We have shown before that liposomes composed of TPE and CHEMS are sensitive to pH below 5.5 (Ellens et al., 1984). When injected into buffers of pH 5.0–4.0 these liposomes aggregate and leak, and we demonstrated that leakage occurs only upon contact between the liposomes. The requirement for contact has been shown before for Ca^{2+}-induced leakage of PS liposomes, and we were therefore interested in whether H^+ would also induce the TPE/CHEMS liposomes to fuse. A mixture of ANTS- and DPX-containing liposomes was injected into buffers of pH 4.0, 4.5, 5.0, and 5.5, and although there is contact-induced leakage from these liposomes (Figure 5A), there is no mixing of aqueous contents (data not shown). We also investigated the behavior of the lipids at low pH using the resonance energy transfer assay (Struck et al., 1981). A 1/9 mixture of NBD-PE/Rh-PE/TPE/CHEMS (0.1/0.1/7/3) and TPE/CHEMS (7/3) liposomes was injected into buffers of pH 4.0, 4.5, 5.0, and 5.5. In Figure 5B it is shown that there is lipid mixing between pH 5.0 and 4.0 but not at pH 5.5. Apparently these liposomes destabilize at low pH, and the bilayers mix. However, the contact-induced bilayer destabilization and lipid mixing do not lead to mixing of aqueous contents.

The TPE/CHEMS liposomes can be induced to fuse and leak with Ca^{2+} at neutral pH (Figure 6A,B). At pH 4.5 the Ca^{2+}-induced fusion is totally inhibited. Interestingly, Mg^{2+} can also induce leakage and fusion of these liposomes (Figure 6D,E), although only at higher concentrations, and there is a lag phase in the induction of fusion. Panels C and F of Figure 6 show the lipid mixing with Ca^{2+} and Mg^{2+}. It is important to note that with 4 mM Mg^{2+}, where there is *no* leakage or mixing of aqueous contents, there is a 35% increase in NBD fluorescence in 3 min.

Discussion

The fusion assay described in this paper monitors membrane fusion through the mixing of aqueous contents of liposomes.

The assay is in principle very similar to the Tb^{3+}/DPA assay (Wilschut & Papahadjopoulos, 1979) and is based on the quenching of ANTS fluorescence by DPX (Smolarsky et al., 1977). Since the quenching reaction is very fast, the assay can be used to continuously monitor the kinetics of mixing of aqueous contents.

ANTS fluorescence is relatively independent of pH down to pH 4.0 (Ellens et al., 1984), and the ANTS/DPX fusion assay can thus be used to monitor directly the effect of H^+ on divalent cation induced fusion of liposomes between pH 7.4 and pH 4.0. Below pH 4.0 ANTS fluorescence is partially quenched due to protonation of the amine group. Since ANTS is still fluorescent at pH 2.0, the ANTS/DPX assay can be used to monitor fusion at this pH, after correcting for fluorescence quenching due to protonation. We can now investigate the ability of H^+ by itself to induce mixing of aqueous contents of PS liposomes.

After addition of Ca^{2+} to a suspension of PS liposomes the ANTS/DPX assay monitors the same sequence of events as is seen with the Tb^{3+}/DPA assay: a rapid mixing of aqueous contents, the rate of which is dependent upon the Ca^{2+} concentration, and a leakage process that is delayed compared to the mixing of aqueous contents. This leakage presumably reflects a collapse of the liposomes into anhydrous cochleates. With Mg^{2+} no mixing of aqueous contents occurs. Although the fusion rates obtained with both assays are comparable, the ANTS/DPX assay gives somewhat faster rates. Since fusion rates are very sensitive to liposome size (Nir et al., 1982; Bentz et al., 1983a, 1985a), we ascribe this difference between the assays to the size distributions of the ANTS and DPX liposomes on the one hand and the Tb^{3+} and DPA liposomes on the other hand.

When PS liposomes are injected into a pH 2.0 buffer, they aggregate and show mixing of aqueous contents. This was indicated by the faster and more pronounced quenching of ANTS fluorescence upon injection of a mixture of ANTS- and DPX-containing liposomes into the pH 2.0 buffer, compared to injection of just ANTS liposomes. Evidence of aqueous contents mixing is provided by the higher level of quenching in the reisolated fused liposomes formed in the mixture compared to quenching in reisolated fused liposomes originating from just ANTS liposomes, 38% and 15%, respectively, while the internal pH in both cases is around 4.0. H^+ is the only monovalent cation to induce membrane destabilization (Nir et al., 1983b), and this is the first time that H^+-induced mixing of aqueous contents has been shown. In terms of bulk concentration H^+ is as effective as Ca^{2+} (10 mM H^+ vs. 10 mM Ca^{2+}), but H^+-induced fusion seems to be more leaky.

Fusion or Destabilization of TPE/CHEMS Liposomes. We have shown before (Ellens et al., 1984) that liposomes composed of TPE and CHEMS are destabilized by H^+ in the range of pH 4.0–5.0, where the CHEMS is protonated and the bilayer is essentially now composed of TPE and cholesterol. The destabilization of the TPE/CHEMS liposomes only occurs after aggregation of the liposomes, and contact between the outer monolayers of the liposomes is necessary for leakage. Here we demonstrate that H^+ also causes mixing of lipids between two apposing TPE/CHEMS bilayers at pH 4.0–5.0 but that it does not lead to mixing of aqueous contents. This is the first lipid system that has been shown to undergo a contact-induced destabilization that leads to leakage and lipid exchange but not mixing of aqueous contents.

It is important to note that the Ca^{2+}-induced leakage of PS liposomes at neutral pH occurs *after* and not during fusion. Close apposition of the bilayers leads to a mixing of aqueous contents, and the initial leakage per fusion event is zero (Bentz et al., 1983a, 1985a). This should be distinguished from the H^+-induced leakage of the TPE/CHEMS liposomes, where bilayer contact does not lead to mixing of contents but directly leads to leakage.

One of the major points of this work concerns the use of the rhodamine/NBD lipid mixing assay as a fusion assay. It is known with some systems that this assay responds similarly to the Tb^{3+}/DPA assay (Hoekstra, 1982; Rosenberg et al., 1983; Wilschut et al., 1983). However, here we have found that this is not always the case [see also Rosenberg et al. (1983) and Ababei & Hildenbrand (1984)]. With H^+ and the TPE/CHEMS liposomes we found lipid mixing and leakage but not mixing of aqueous contents. This is a clear example of contact-induced lysis where the protonated CHEMS/TPE does not re-form a bilayer structure around the area of contact and destabilization, although there is substantial lipid mixing. More noteworthy are the cases of low Ca^{2+} (2 mM) and Mg^{2+} (4 mM) concentrations with the TPE/CHEMS liposomes where there was neither fusion nor leakage, but there was substantial energy transfer from the NBD/rhodamine assay (Figure 6). There are two possible mechanisms for this event. First, there may be mixing of only the outer monolayers of the liposomes while the inner monolayers remain intact and thus prevent the mixing of contents or leakage. Second, there may be probe exchange (the NBD and/or the rhodamine) between the liposomes at the points of contact without the concomitant mixing of the unlabeled lipids. Which of these mechanisms is responsible for the observed fluorescence signal changes is not known at present. In any event, it is clear that the NBD/rhodamine lipid mixing assay cannot be used as an unambiguous fusion assay without the concomitant use of an aqueous contents assay system.

Comparison of CHEMS and PS. At neutral pH CHEMS acts very much like PS. It stabilizes TPE in a bilayer structure, and TPE/CHEMS liposomes can be induced to fuse and leak with both Ca^{2+} and Mg^{2+}. These fusion processes are quite similar to Ca^{2+}- and Mg^{2+}-induced fusion of PS/PE (1/3) liposomes; even the bulk concentrations of the divalent cations needed to induce fusion are about the same (Düzgüneş et al., 1981). Since it is known that the fusion of PS-containing liposomes depends on the amount of Ca^{2+} bound per PS head group, we presume that Ca^{2+}-, or Mg^{2+}-, induced fusion of TPE/CHEMS liposomes works the same way. Likewise, there is a minimal number of Ca^{2+} bound per PS necessary to produce a threshold fusion reaction once the bilayers are closely apposed (Bentz et al., 1983b; Bentz & Düzgüneş, 1985). The Ca^{2+}-induced fusion of TPE/CHEMS liposomes is totally inhibited at pH 4.5, suggesting that at this pH the protons have competed a sufficient number of Ca^{2+} ions off of the CHEMS to eliminate the fusion reaction.

Conclusion

H^+ induces fusion of PS liposomes and leakage from PE/CHEMS liposomes. Apparently, the H^+-induced close apposition and destabilization of the PE/CHEMS bilayers do not lead to a coalescence of the aqueous compartments, and it is conceivable that the leakage occurs around the edges of the contact area. With PS liposomes the H^+-induced close apposition and destabilization do lead to coalescence of aqueous compartments, so that there is a reorganization of the lipids in the contact area that allows mixing of contents. It is necessary for fusion that after the destabilization of the apposed bilayers a new bilayer, encapsulating the mixed contents, is formed. It is known that the PE/CHEMS lipid system can undergo a lamellar–H_{II} phase transition in the presence of H^+

and Ca^{2+} (Lai et al., 1985). It may well be that the H^+-induced leakage that is observed with the PE/CHEMS liposomes is due to a collapse of the lipids into H_{II} phase like tubes, starting from aggregation of inverted micelles formed between apposing monolayers (Siegel, 1984). The H_{II} phase tubes would not allow mixing of contents but would lead to lipid mixing and leakage.

Added in Proof

We have now found that the TPE/CHEMS (7/3) liposomes undergo the lamellar–H_{II} phase transition at pH 4.5 over the temperature range of T_H = 23–37 °C. The phase transition is not required to induce leakage, which will occur at 10–15 °C; however, the initial kinetics of leakage and lipid mixing are vastly enhanced at temperatures at and above T_H. Furthermore, there is *no* mixing of aqueous contents induced by H^+ at any temperature in the range of 10–35 °C (Bentz et al., 1985b). Also, it has been found recently that PE/oleic acid MLV show mixing of aqueous contents at low pH with the ANTS/DPX assay (Düzgüneş et al., 1985).

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Registry No. CHEMS, 1510-21-0; ANTS, 117-42-0; DPX, 14208-10-7; Ca, 7440-70-2; hydrogen ion, 12408-02-5.

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