Modulation of Membrane Fusion by Asymmetric Transbilayer Distributions of Amino Lipids†

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ABSTRACT: The fusion of model lipid bilayers containing synthetic amino lipids and the regulation of this fusion by inducing transbilayer asymmetry of these amino lipids via imposed pH gradients are demonstrated. Liposomes of 100 nm diameter consisting of 5 mol % 1,2-dioleoyl-3-(*N*,*N*-dimethylamino)propane (**AL1**) in a mixture of egg phosphatidylcholine (EPC), dioleoylphosphatidylethanolamine (DOPE), and cholesterol in a ratio of 35:20:45 do not fuse at pH 4.0. Fusion also is not observed upon increasing the external pH of these vesicles to 7.5, which results in the rapid transport of **Al1** to the inner monolayer, as measured by a fluorescent probe sensitive to surface charge. However, dissipation of the imposed pH gradient leads to redistribution of **AL1** to the outer monolayer at pH 7.5 and causes liposomal fusion, as detected by fluorescent lipid-mixing assay and freeze-fracture electron microscopy. The effect of varying the hydrocarbon structure of **AL1** on the rate of fusion is demonstrated with five synthetic analogues, **AL2**–**AL6**. Higher rates of fusion occur with lipids containing longer unsaturated acyl chains and with lower values of pK_a for the membrane-bound amino lipids. Fusion is also associated with destabilization of the bilayer at pH 7.5, as indicated by the formation of the hexagonal H_{II} phase.

Membrane fusion is a fundamental phenomenon in cell biology. Fusion is involved in intracellular processes, such as endocytosis, exocytosis, and intracellular trafficking and sorting events, as well as intercellular processes such as fertilization. While membrane fusion proteins, such as those associated with the membranes of enveloped viruses, play vital roles in mediating fusion (White, 1992), the lipid composition of the fusing membranes is also a critical determinant. Studies employing lipid vesicle systems have shown a strong correlation between the tendency of vesicles to fuse and the propensity of the component lipids to adopt non-bilayer phases, such as the hexagonal H_{II} organization (Cullis & de Kruijff, 1979). This has led to the suggesting that non-bilayer lipid structures such as inverted micelles or other bilayer defects are intermediary structures in fusion events (Cullis & Hope, 1978). The direct influence of lipid composition on membrane fusion suggests that regulation of the transbilayer distribution of "fusogenic" lipid species can also regulate membrane fusion. This has been demonstrated using vesicles containing phosphatidic acid (PA), where fusion stimulated by Ca^{2+} is reduced proportionately when the PA is sequestered to the inner monolayer (Eastman et al., 1992).

In this work, we describe large unilamellar vesicle (LUV) systems that do not fuse when the fusogenic lipids are sequestered on the inner monolayer, but that do fuse when the fusogenic lipids appear in the outer monolayer, in the absence of external stimuli such as Ca^{2+}. This work is based on previous studies showing that unsaturated diglycerides are extremely potent fusogens (Das & Rand, 1986) and that uncharged lipids are more able to adopt or induce non-bilayer structures than their charged forms (Cullis et al., 1986). In particular, it was reasoned that a diglyceride substituted with an amino function should be highly fusogenic at pH values above the pK_a of the conjugate acid of the amine, while at lower pH values the presence of headgroup charge would be more compatible with bilayer structure. Further, the presence of the amino function should allow the amino lipid to be sequestered on the inner monolayer of LUV with an acidic interior, as has been previously demonstrated with stearylamine (Hope & Cullis, 1987).

We have synthesized a number of amino lipids that have behavior consistent with these expectations. These synthetic compounds exhibit varying degrees of fusogenicity, which correlate with their chemical structure and acid–base properties. Of particular interest is a preparation of 5 mol % 1,2-dioleoyl-3-(*N*,*N*-dimethylamino)propane (**AL1**) in LUVs consisting of egg phosphatidylcholine (EPC), dioleoylphosphatidylcholine (DOPE), and cholesterol (Chol) (EPC/DOPE/Chol, 35:20:45). In these systems, it is shown that **AL1** can be sequestered on the inner monolayer by a pH gradient (pH_i 4.0, pH_o 7.5), and dissipation of the pH gradient results in the outward movement of **AL1** and extensive liposomal fusion.

MATERIALS AND METHODS

Lipids and Chemicals. Egg phosphatidylcholine (EPC), dioleoylphosphatidylethanolamine (DOPE), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dioleoyl-*sn*-phosphatidylethanolamine (NBD-PE), and *N*-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-*sn*-phosphatidylethanolamine (Rh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). Aminonaphthalenetrisulfonic acid (ANTS) and *p*-xylylenebis(pyridinium) bromide (DPX) were purchased from Molecular Probes (Eugene, OR). Oleic acid, cholesterol (Chol), nigericin, potassium 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS), and all buffers were supplied by Sigma Chemical Co. (St. Louis, MO). 3-(*N*,*N*-Dimethylamino)-1,2-propanediol, oxalyl chloride, acetyl chloride, butyric acid, and decanoyl chloride were purchased from Aldrich Chemical Co. (Milwaukee, WI), and oleic acid-*9,10-d*$_2$ was supplied by Cambridge Isotope Laboratories (Woburn, MA). Organic solvents were all HPLC grade and were used without redistillation.

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Synthesis of ±-1,2-Dioleoyl-3-(N,N-dimethylamino)propane (**AL1**). This compound was prepared by the method of Leventis and Silvius (1990). Three milliliters (35 mmol) of oxalyl chloride was added to 1.0 g (3.5 mmol) of oleic acid dissolved in 10 mL of benzene and stirred at room temperature for 1 h. After the removal of solvent and excess oxalyl chloride under vacuum, the acid chloride was dissolved in 5 mL of diethyl ether, and an additional 5 mL of ether containing 0.20 g (1.7 mmol) of 3-(*N,N*-dimethylamino)-1,2-propanediol and 0.15 g of pyridine was added. The resulting mixture was stirred at room temperature for 30 min before quenching with 1 mL of methanol and removing solvents under vacuum. The crude product was dissolved in 50 mL of hexane and washed with 2 × 25 mL of 0.1 M potassium hydroxide in methanol/water (1:1) followed by 25 mL of 0.1 M aqueous sodium chloride. Drying over anhydrous sodium sulfate and removal of hexane under vacuum gave a slightly yellow oil. Column chromatography on silica gel (70–230 mesh), eluting with ethyl acetate, gave 0.92 g (84%) of pure product (TLC, R_f = 0.5). The structure of the product was confirmed by 200 MHz ^{1}H NMR. Similar procedures were used to prepare the deuteriated analogue, ±-1,2-bis(9′,10′-dideuteriooleoyl)-3-(*N,N*-dimethylamino)propane (**AL1**-d_4), and ±-1,2-didecanoyl-1-(*N,N*-dimethylamino)propane (**AL-6**). See Figure 1 for structures.

Synthesis of ±-1-Oleoyl-2-hydroxy-3-(N,N-dimethylamino)propane (**AL2**) *and Asymmetric ±-1,2-Diacyl-3-(N,N-dimethylamino)propanes* (**AL3**–**AL5**). Oleoyl chloride (3.5 mmol), prepared as described above, was dissolved in 5 mL of THF and added to a 5-fold excess of 3-(*N,N*-dimethylamino)-1,2-propanediol (2.0 g, 17 mmol) and 0.15 g of pyridine in 25 mL of THF at 0 °C. Crude 1-monooleyl-2-hydroxy-3-(*N,N*-dimethylamino)propane (**AL2**) was isolated as described above and purified by column chromatography on silica gel using ethyl acetate/methanol (3:1) as the eluant (R_f = 0.4). Subsequent acylation with 1 equiv of acetyl chloride, butyryl chloride, or decanoyl chloride with the reaction conditions, extraction procedures, and purification as described above produced **AL3**, **AL4**, or **AL5**, respectively. For structures see Figure 1.

Preparation of LUVs. Chloroform solutions of lipids were dried by vortex-mixing under nitrogen followed by the removal of residual solvent under high vacuum for 1 h. The resulting lipid films were hydrated by vortex-mixing with appropriate buffers to produce multilamellar vesicles (MLVs). Five freeze–thaw cycles were used to achieve homogeneous mixtures. The MLVs were extruded 10 times through two 100 nm pore size polycarbonate filters to produce LUVs.

Determination of pK_a for Amino Lipids in Lipid Vesicles. To determine the pH at which the synthetic amino lipids **AL1**–**AL6** in liposomal membranes lose their positive charge, LUVs containing 0 and 10 mol % of each amino lipid in EPC/Chol (55:45) were prepared in 5 mM HEPES, 5 mM ammonium acetate, 5 mM KCl, and 1 μM nigericin at pH 7.5. Preparations were diluted to 25 μM total lipid in 5 mM HEPES, 5 mM ammonium, 5 mM KCl, and 2 mM TNS at pH's ranging from 3.0 to 10.0. The surface charge was monitored by determining the TNS fluorescence at each pH, which was measured on a Perkin Elmer LS-50 spectrofluorometer using excitation and emission wavelengths of 321 and 445 nm, respectively.

Determination of Lipid Asymmetry by TNS Fluorescence. The transport of **AL1** to the inner monolayer of vesicles and the subsequent redistribution to the outer monolayer were demonstrated by TNS fluorescence using a procedure adapted from Eastman et al. (1991). EPC/Chol (55:45) vesicles were prepared with 0 and 10 mol % **AL1** in either 300 mM citrate (pH 4.0) or 20 mM HEPES and 150 mM NaCl (pH 7.5). For the samples prepared at low pH, the external buffer was exchanged for 300 mM sucrose and 1 mM citrate (pH 4.0) on Sephadex G-25 columns. All samples were diluted to 30 mM total lipid. TNS fluorescence time courses were performed over 200 s as follows. At 10 s, 10 μL of lipid was added to 3 mL of buffer containing 20 mM HEPES, 150 mM NaCl, and 5 μM TNS, at pH 7.5. At 100 s, 30 μL of 10 μM nigericin was added.

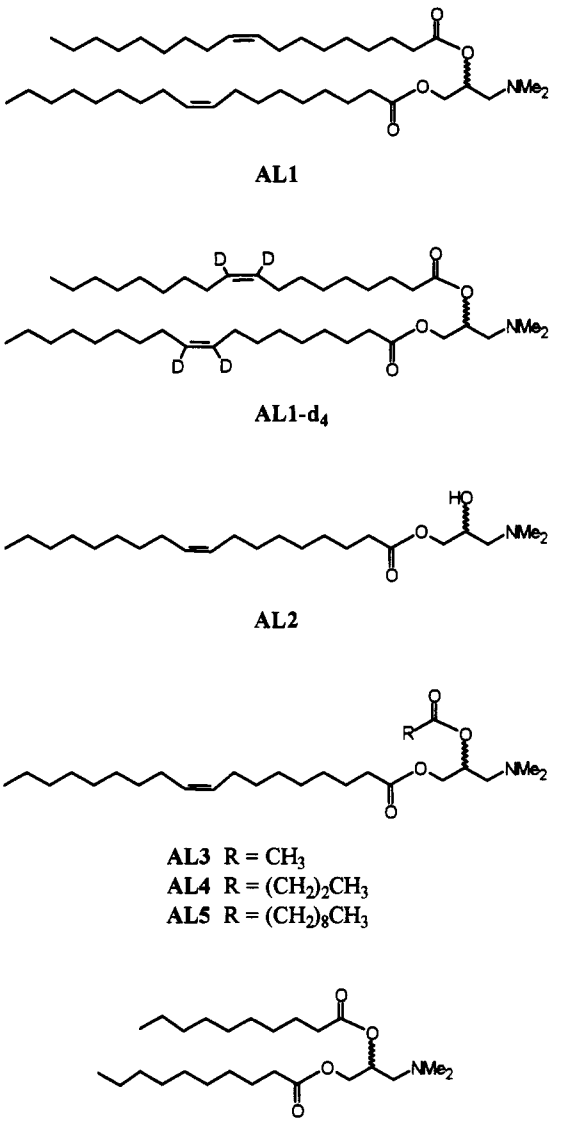

FIGURE 1: Structures of the synthetic amino lipids used in this study. All compounds are based on 3-(*N,N*-dimethylamino)-1,2-propanediol and are optical racemates, as indicated by the jagged bond at the 2-position of the aminoglycol backbone.

Lipid-Mixing Fusion Assay. Fusion was monitored by the decrease in resonance energy transfer (RET) resulting from fluorescent probe dilution, as described by Struck et al. (1981). LUVs of a desired composition were prepared in 300 mM citrate (pH 4.0). Similar vesicles containing 0.5 mol % of both NBD-PE and Rh-PE were also prepared. External buffer was exchanged for 1 mM citrate and 300 mM sucrose (pH 4.0) on Sephadex G-25 columns before diluting preparations to 10 mM lipid. For fusion assays, 20 μL of fluorescently labeled vesicles and 60 μL of unlabeled vesicles were added

to 3.92 mL of 20 mM HEPES and 150 mM NaCl (pH 7.5). After 5 min of incubation at room temperature, a 3 mL aliquot was added to a cuvette, and the fluorescence was monitored over time. Excitation and emission wavelengths were 465 and 535 nm, respectively, and a 530 nm emission cutoff filter was used. To dissipate the gradient and induce fusion, 100 μL of 3.0 M ammonium acetate was added at 30 s.

Each fusion time course was normalized using three control curves: (i) an assay excluding unlabeled vesicles (F_{nu}) to account for the effect of pH gradient dissipation on fluorescence due to lipid charge interactions; (ii) a maximum fluorescence trace (F_{max}) measured after adding 100 μL of 25 mM Triton X-100; and (iii) a zero-fusion trace (F_0) in which no ammonium acetate was added. The percent change in fluorescence ($\%\Delta F/\Delta F_{max}$) was then calculated as

$$\% \frac{\Delta F}{\Delta F_{max}} = \left(\frac{F - F_{nu}}{F_{max} - F_0}\right)100$$

for each point in the fluorescence time course. Liposomes containing both fluorescent probes at one-quarter the concentration used in the assay gave $\Delta F/\Delta F_{max} = 80\%$, and the fusion results presented were not corrected to reflect the increase observed upon the addition of Triton X-100.

Contents-Mixing Fusion and Leakage Assays. Liposomes were prepared with either 25 mM ANTS and 300 mM citrate (pH 4.0) or 100 mM DPX and 300 mM citrate (pH 4.0). External buffer was exchanged with 1 mM citrate and 300 mM sucrose (pH 4.0), as described above, and the preparations were diluted to 10 mM total lipid. To detect contents mixing, 40 μL of the ANTS preparation was combined with 120 μL of the DPX preparation and made up to 4 mL with 20 mM HEPES and 150 mM NaCl (pH 7.5). The quenching of ANTS by DPX was monitored by measuring the ANTS fluorescence of a 3 mL aliquot over 30 min. Excitation and emission wavelengths were 360 and 530 nm, respectively, and a 490 nm emission cutoff filter was used. To dissipate pH gradients and induce fusion, 100 μL of 3.0 M ammonium acetate was added at 30 s. Maximum quenching was determined by repeating the assay with an equivalent amount of liposomes containing 6 mM ANTS, 75 mM DPX, and 300 mM citrate (pH 4.0). Zero quenching was measured using only ANTS liposomes. Leakage upon dissipation of the pH gradient was determined by comparing the result obtained above with the ANTS/DPX liposomes with the results of a similar assay in which 50 μL of 100 Triton X-100 was added at 30 s.

^{31}P NMR and ^{2}H NMR Spectroscopy. Freeze–thawed MLVs of 5 mol % **AL1**-d_4 in EPC/DOPE/Chol (35:20:45) were prepared in 20 mM HEPES, 20 mM ammonium acetate, and 150 mM NaCl at pH 4.0 and 7.5 in deuterium-depleted water. Sample concentrations were approximately 200 mM total lipid. High-resolution ^{31}P spectra were recorded at 81.02 MHz on a Bruker MSL200 spectrometer, using a 2.8 μs pulse and a 1 s repeat. Temperature was maintained at 23 °C with a liquid nitrogen flow system. The FID was accumulated over 1000 scans and transformed with 50 Hz line-broadening. ^{2}H NMR broad-line spectra were recorded at 46.175 MHz on a home-built 300 MHz spectrometer using a 5 μs 90° pulse, 50 μs interpulse spacing, 30.5 μs ring-down delay, and a 300 ms repetition time. A quadrupole echo sequence with eight-step phase cycling was used to accumulate 200 000 scans. The resulting free induction decay (FID) was transformed with 100 Hz line-broadening.

Freeze-Fracture Electron Microscopy. LUVs consisting of 5 mol % **AL1** in EPC/DOPE/Chol (35:20:45) were prepared as above in 300 mM sodium citrate at pH 4.0, with a total lipid concentration of 100 mM. External buffer was exchanged for 1 mM citrate and 300 mM sucrose (pH 4.0) on a Sephadex G-25 column before diluting the preparation to approximately 10 mM lipid with 20 mM HEPES and 150 mM NaCl (pH 7.5). After the removal of a control sample, the pH gradient was dissipated by the addition of 3 M ammonium acetate (pH 7.5) to a final concentration of 100 mM. Platinum/carbon replicas were prepared as described previously (Fisher & Branton, 1974) for samples at 4, 15, and 30 min after dissipation of the gradient, as well as the control with the pH gradient present.

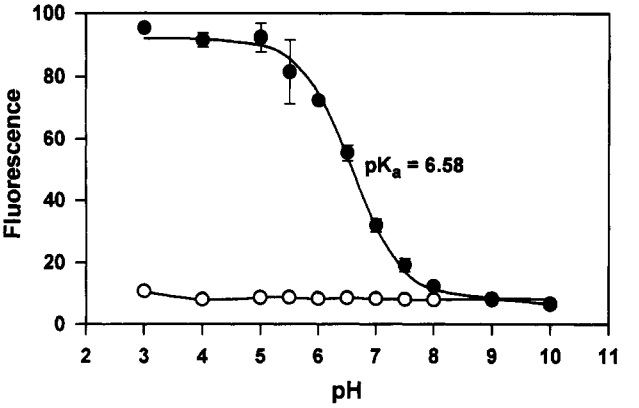

FIGURE 2: Effect of pH on the TNS fluorescence of EPC/Chol (55:45) vesicles containing 0 mol % (○) and 10 mol % (●) **AL1**. Liposomes were prepared in 5 mM HEPES, 5 mM ammonium acetate, and 1 μM nigericin at pH 4.0. Preparations were diluted to 25 μM total lipid in the same buffer containing 2 μM TNS at various pH's. Data shown represent mean values and standard deviations from duplicate experiments. Error bars are smaller than the symbols unless indicated otherwise.

RESULTS

Effects of pH on Amino Lipid Charge. The amino lipids would be expected to be more fusogenic in their neutral forms, which predominate at pH values above the pK_a of the protonatable amino group. Alternatively, in order to sequester the amino lipid to the acidic interior, the lipid must be charged at the interior pH. The pK_a's of the various amino lipids in a lipid bilayer were determined by measuring the relative surface charges as a function of pH by TNS fluorescence. A pH titration curve of 10 mol % **AL1** in EPC/Chol (55:45) vesicles is shown in Figure 2. Fluorescence was measured in the absence of pH gradients. Fitting the data to the Henderson–Hasselbalch equation indicates a pK_a of 6.58 for the conjugate acid of **AL1**. At pH 4.0, virtually all of the amino lipid is in the charged form, while at pH 7.5 approximately 90% is in the neutral form. These results suggest that the acid–base properties of **AL1** are ideal for the induction of asymmetry by a pH gradient (pH_i 4.0, pH_0 7.5). In addition, the prevalence of the neutral form at pH 7.5 should provide the bilayer destabilization desired to induce membrane fusion.

A comparison of results derived from similar pH titration curves for the synthetic amino lipids **AL2**–**AL6** is given in Table 1. The effects of chemical structure on the acid–base characteristics of the amines, when incorporated into liposomes, are manifested as changes in the pK_a values observed and in the fraction of amino lipid species remaining charged at pH 7.5. These changes likely stem from the depth of penetration of the amine headgroup into the lipid bilayer, as influenced by the relative hydrocarbon chain content and the

Table 1: Apparent Acid Dissociation Constants (pK_a) and Percent of Charged Species at pH 7.5 for Amino Lipids **Al1–AL6**[a]

amino lipid	apparent pK_a	% charged at pH 7.5
AL1	6.58	11
AL2	7.57	54
AL3	6.79	16
AL4	6.66	13
AL5	6.73	14
AL6	6.81	17

[a] The data were derived from TNS fluorescence titrations as shown in Figure 3. Curves for each amino lipid were fit to the Henderson–Hasselbalch equation by iteratively varying pK_a and maximum and minimum fluorescence.

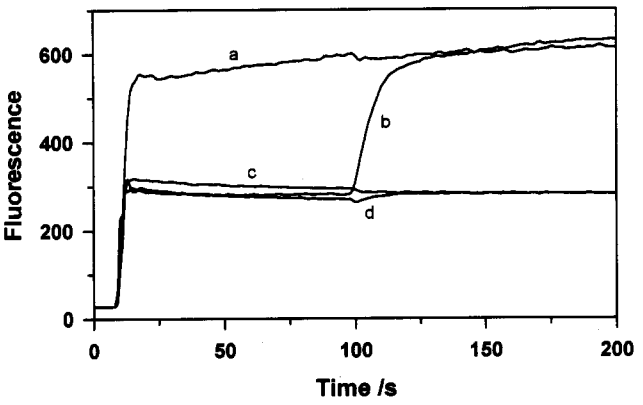

FIGURE 3: TNS fluorescence traces showing the effect of induction and dissipation of a pH gradient on **AL1** in EPC/Chol (55:45) vesicles. At 10 s, liposomes prepared with either 10 mol % (a, b) or 0 mol % (c, d) **AL1** were added to buffer containing 20 mM HEPES, 150 mM NaCl, and 5 μM TNS (pH 7.5) to a final lipid concentration of 0.1 mM. Internal buffer was either 20 mM HEPES buffer and 150 mM NaCl (pH 7.5) (a, c) or 300 mM citrate (pH 4.0) (b, d). At 100 s, nigericin was added to a final concentration of 0.1 μM.

polarity of the headgroup. Compound **AL2**, having only a single oleoyl chain and an unsubstituted hydroxy group, has a pK_a of 7.57, compared to 6.58 for **AL1** with two oleoyl chains. Therefore, at pH 7.5 the **AL2** headgroup remains more than 50% charged. Esterification of the hydroxyl at the 2-position with acetate to give **AL3** reduces the pK_a to 6.79, and the fraction of charged species at pH 7.5 is only 16% compared to 11% for **AL1**. The remaining compounds, **AL4–AL6**, also have sufficient hydrocarbon content to have pK_a's and relative charges at pH 7.5 approaching those of **AL1**. The influence of the structural differences of these amino lipids on their fusogenic capacity will be discussed in following sections.

Asymmetry of Amino Lipids in Liposomes. The effect of a pH gradient on the transbilayer distribution of the synthetic amino lipids was demonstrated by monitoring the changes in the surface of vesicles comprising amino lipids and EPC/Chol (55:45) by TNS fluorescence. Vesicles with an internal pH of either 4.0 or 7.5 were injected into pH 7.5 buffer to give the fluorescence time courses shown in Figure 3. Trace a is the fluorescence of 10 mol % **AL1** in the absence of a gradient, with internal and external pH 7.5. Trace b is the same lipid composition with an initial internal pH of 4.0. The pH gradient produced upon the addition of these vesicles to buffer at pH 7.5 results in an immediate attenuation of fluorescence relative to trace a. When the pH gradient is dissipated by the addition of nigericin, the fluorescence in b increases to the value observed in a. Nigericin is used to dissipate the gradient in this case, since TNS fluorescence is highly sensitive to shielding of the membrane surface charge by salts. Preparations containing no amino lipid either in the absence (c) or presence (d) of a

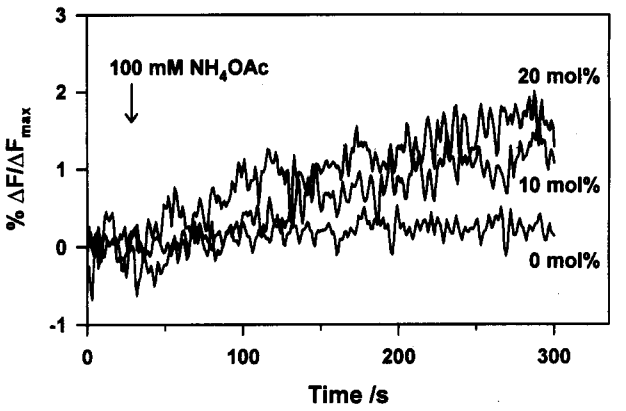

FIGURE 4: Effect of **AL1** concentration (0–20 mol %) on the fusion of EPC/Chol (55:45) vesicles by RET fluorescent probe dilution. Vesicles of each composition were prepared with and without 0.5 mol % each of NBD-PE and Rh-PE, and internal buffer of 300 mM sodium citrate (pH 4.0), and an external buffer of 20 mM HEPES and 150 mM NaCl (pH 7.5). The labeled and unlabeled vesicles were mixed in a 1:3 ratio and diluted to 0.20 mM total lipid. The pH gradient was dissipated by the addition of ammonium acetate to a final concentration of 100 mM at 30 s.

pH gradient exhibited the same surface charge as those containing 10 mol % **AL1** with a pH gradient. Note that the differences in fluorescence observed for liposomes containing **AL1** reflect only the approximately 10% of the amino lipid that remains charged at pH 7.5.

Comparison of these traces clearly shows that the imposition of a pH gradient (pH_i 4.0, pH_0 7.5) causes rapid and complete loss of positive surface charge and, therefore, **AL1** from the outer monolayer. Subsequent dissipation of the gradient results in the redistribution of the amino lipid between the bilayers, accompanied by an increase in surface charge. Similar behavior was observed for amino lipids **AL2–AL6** (data not shown).

Fusion of Liposomes Containing AL1. The dissipation of the pH gradient across the membranes of vesicles with acidic interiors can also be achieved by the addition of ammonium ions, which cross the membrane in the form of neutral ammonia to become protonated in the vesicle interior, thus raising the internal pH. As demonstrated above, the LUVs containing amino lipids primarily on the inner monolayer, the loss of the pH gradient causes a loss of transbilayer asymmetry accompanied by neutralization of the amino lipid. Membrane fusion resulting from this redistribution was monitored by a loss in RET between the fluorescently labeled lipids, NBD-PE and Rh-PE. When vesicles containing both of these labels fuse with unlabeled vesicles, the dilution of the fluorescent probes results in increased fluorescence for NBD-PE. Appreciable exchange of these labeled lipids between vesicles does not appear to occur even in aggregated systems, and fluorescence increases only upon the mixing of membrane lipids (Hoekstra, 1982).

The use of this fluorescent probe dilution assay to demonstrate fusion in LUVs containing 0–20 mol % **AL1** is depicted in Figure 4. Unlabeled and labeled vesicles (3:1) consisting of EPC/Chol (55:45) without **AL1** showed no increase in fluorescence upon dissipation of the pH gradient. With 10 and 20 mol % **AL1**, only small increases in $\Delta F/\Delta F_{max}$ are observed (less than 2% over 5 min). This represents very limited lipid mixing. Complete fusion under these conditions would give a $\Delta F/\Delta F_{max}$ of about 80%, as determined by preparing vesicles with the fluorescent labels at one-quarter of the assay concentration. The low rates of increase in fluorescence observed indicate that the ability of **AL1** to induce

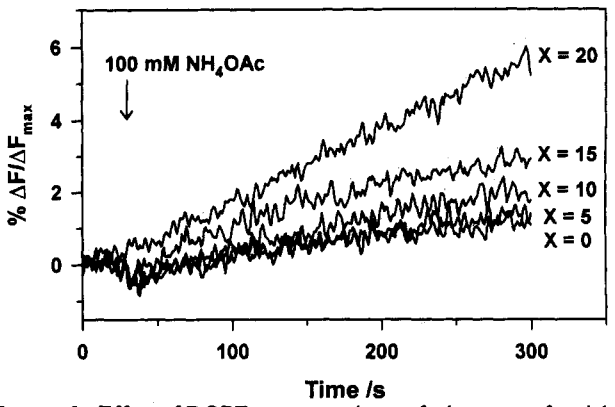

FIGURE 5: Effect of DOPE concentration on fusion rates of vesicles containing **AL1**. LUVs were prepared with EPC/DOPE/Chol (55 – *X*:*X*:45, *X* = 0, 5, 10, 15, 20), 10 mol % **AL1**, with or without 0.5 mol % each of NBD-PE and Rh-PE, an internal buffer of 300 mM sodium citrate (pH 4.0), and an external buffer of 20 mM HEPES and 150 mM NaCl (pH 7.5). Fusion assays were carried out as described in Materials and Methods.

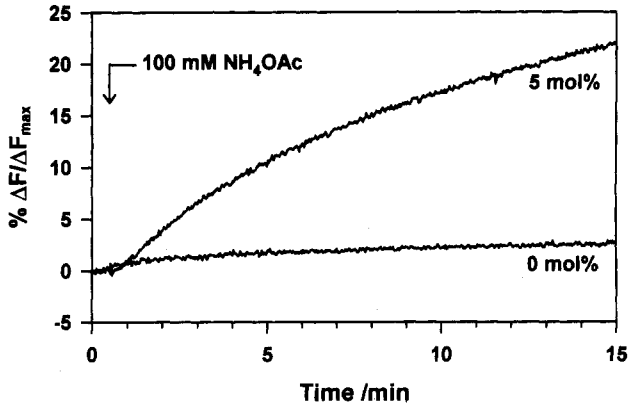

FIGURE 6: Fusion of vesicles consisting of 0 and 5 mol % **AL1** in EPC/DOPE/Chol (35:20:45). LUVs were prepared with or without 0.5 mol % each of NBD-PE and Rh-PE, an internal buffer of 300 mM sodium citrate (pH 4.0), and an external buffer of 20 mM HEPES and 150 mM NaCl (pH 7.5). Fusion assays were carried out as described in Materials and Methods.

pH gradient-controlled fusion in EPC/Chol vesicles is limited. For **AL1** concentrations greater than 20 mol % in EPC/Chol vesicles, rapid and complete aggregation was observed following extrusion at pH 4.0, making it impossible to study lipid asymmetry and fusion in such systems.

The rates of fusion achieved with vesicles containing **AL1** were improved by the addition of DOPE to the lipid preparation. DOPE provides additional membrane destabilization by virtue of its preference for the hexagonal H_{II} phase. Fusion assays for EPC/DOPE/Chol vesicles containing 10 mol % **AL1**, where increasing levels of DOPE replace the EPC, are shown in Figure 5. Increases in fusion rates were evident with increasing DOPE concentrations up to a ratio of 35:20:45 (EPC/DOPE/Chol). This formulation gave a $\Delta F/\Delta F_{max}$ of greater than 5% after 5 min. However, at this level of DOPE, some aggregation of vesicles was observed at low pH. Higher levels of DOPE again gave rapid aggregation.

The best rates of fusion were observed when the **AL1** concentration was reduced to 5 mol % in EPC/DOPE/Chol (35:20:45), as shown in Figure 6. At pH 4.0, this formulation produces stable vesicles that remain stable when the external pH is increased to 7.5. Dissipation of the pH gradient results in a nearly linear increase in $\Delta F/\Delta F_{max}$ over the first 5 min to a value greater than 10%. As expected for a liposomal fusion event, the rate of fusion decreases with time, but fusion clearly continues for the duration of the 15 min assay.

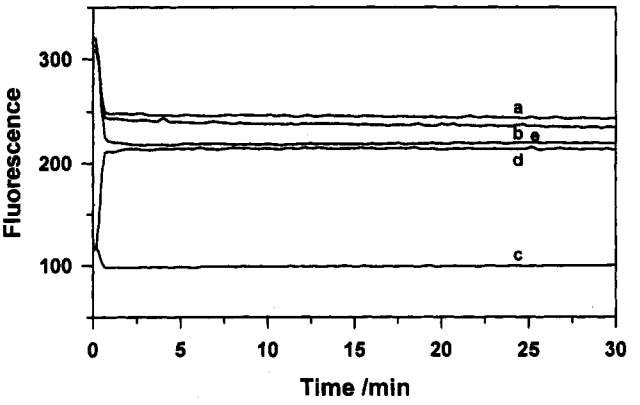

FIGURE 7: ANTS/DPX contents mixing assays for liposomes consisting of 5 mol % **AL1** in EPC/DOPE/Chol (35:20:45). (a) LUVs containing 300 mM citrate (pH 4.0) and either 25 mM ANTS or 100 mM DPX were mixed in a ratio of 1:3 (ANTS:DPX) in 20 mM HEPES and 150 mM NaCl (pH 7.5) to a final lipid concentration of 0.4 mM. The pH gradient was dissipated by the addition of ammonium acetate to a concentration of 100 mM at 30 s, and ANTS fluorescence was monitored over 30 min. (b) Control curve with no DPX liposomes, representing zero contents mixing. (c) Liposomes containing 6 mM ANTS and 75 mM DPX, representing maximum contents mixing. (d) Same as c, with the addition of Triton X-100 to a final concentration of 1.6 mM, representing maximum leakage. (e) Same as a, with the addition of Triton X-100 to demonstrate fluorescence equal to d.

The liposomal fusion observed in this lipid system by fluorescent lipid probe dilution could not be confirmed by the ANTS/DPX contents mixing assay (Ellens et al., 1984). Dissipation of the pH gradient with ammonium acetate gave a rapid attenuation of ANTS fluorescence both in the presence and absence of DPX liposomes (Figure 7). This pH sensitivity may result from a change in the interaction of ANTS with **AL1** or the inner liposomal monolayer upon neutralization and redistribution of the amino lipid. After this initial loss of fluorescence and up to 30 min, there was no further decrease in ANTS fluorescence, indicating fusion with DPX liposomes. In fact, the mixing assay gave a slight increase in fluorescence relative to the control assay with no DPX liposomes, which is the opposite of the expected result. On the other hand, dissipation of the gradient for liposomes containing both ANTS and DPX gave, after the initial attenuation, no increase in fluorescence over 30 min, suggesting that leakage of liposomal contents also does not occur. Therefore, while liposomal fusion could not be confirmed with this contents mixing assay, the lipid mixing that was shown to occur does not appear to be a result of membrane rupture. The terbium–dipicolinic acid (Tb/DPA) assay, which has also been used for contents mixing, cannot be applied for liposomes with acidic interiors (Ellens et al., 1985).

^{31}P NMR and ^{2}H NMR Spectroscopy. The changes in phospholipid morphology that accompany the bilayer destabilization, leading to the fusion of vesicles containing **AL1**, were investigated by high-resolution phosphorus-31 NMR. MLVs were prepared by incorporating 5 mol % of the deuterium chain-labeled analogue **AL1**-d_4 into EPC/DOPE/Chol (35:20:45) MLVs. The use of MLVs in this experiment avoids the loss of useful information caused by rapid tumbling of LUVs on the NMR time scale. Differences in morphology of the phospholipids, EPC and DOPE, at pH 4.0 and 7.5 are evident in the spectra shown in Figure 8a. At pH 4.0, a typical bilayer signal with an upfield peak and a downfield shoulder is observed. At pH 7.5, the bilayer signal is reduced slightly, and a hexagonal H_{II} signal is seen as a more intense downfield shoulder. The pH-induced destabilization of these vesicles

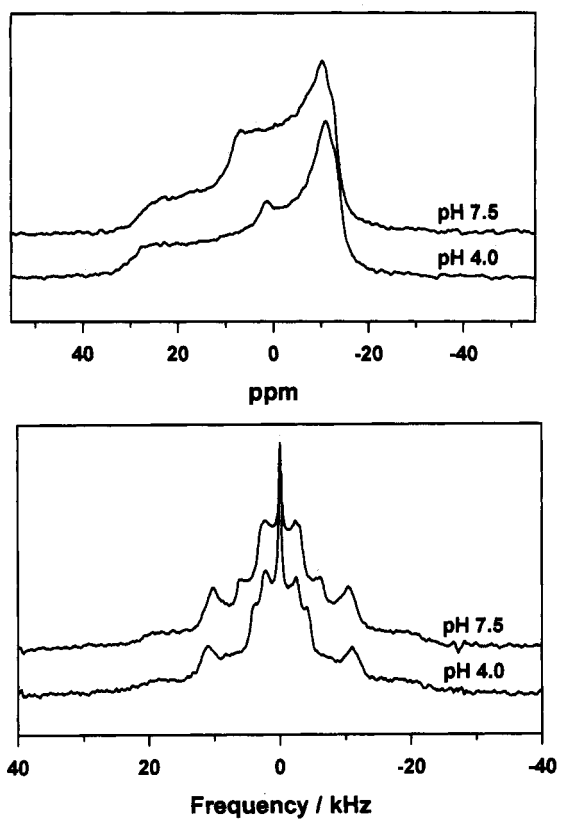

FIGURE 8: (a, top) ^{31}P NMR and (b, bottom) ^{2}H NMR spectra of freeze–thawed MLVs prepared with 5 mol % **AL1**-d_4 in EPC/DOPE/Chol (35:20:45) buffered with 20 mM HEPES, 20 mM ammonium acetate, and 150 mM NaCl at pH 4.0 and 7.5.

clearly involves morphological changes for at least some of the phospholipid in the bilayer.

The accompanying changes in the phase behavior of **AL1**-d_4 can be seen in the quadrupolar deuterium spectra given in Figure 8b. At pH 4.0, the ^{2}H spectrum shows deuterium splittings for the olefinic deuterons in the range expected for oleoyl chains on a lipid incorporated into a bilayer. The shoulders on the peaks arise from the nonidentical chains on the 1- and 2-positions of the aminoglycol backbone, and the strong central isotropic signal probably results from the presence of smaller vesicles. In the sample prepared at pH 7.5, the bilayer signal persists, although it appears to be slightly less ordered, judging from the decrease in quadrupolar splittings. The amino lipid clearly remains associated with the bilayer. In addition, there is a distinct signal with a splitting of about 12 kHz, which we believe arises from the portion of the amino lipid in the hexagonal H_{II} phase. The appearance of this signal is again consistent with the destabilization of the bilayer, leading to membrane fusion.

Freeze-Fracture Electron Microscopy. Additional evidence of the fusion of vesicles consisting of 5 mol % **AL1** in EPC/DOPE/Chol (35:20:45) is given by the freeze-fracture electron micrographs in Figure 9. Vesicles with the amino lipid sequestered on the inner monolayer prior to dissipation of the pH gradient (pH_i 4.0, pH_o 7.5) appear as well-defined individual spheres. Four minutes after the dissipation of the gradient by ammonium acetate, large aggregates and fused vesicle are already visible. This fusion process continues, and by 30 min much of the lipid in the sample is found in very large fused vesicles.

Effects of Amino Lipid Structure. A comparison of the fusogenic capacity of the amino lipids **AL1**–**AL6** was made by assaying for fusion with vesicles consisting of 5 mol % of each amino lipid in EPC/DOPE/Chol (35:20:45), as shown in Figure 10. **AL2**, which has a single oleoyl chain, gives very little liposomal fusion at pH 7.5 compared to liposomes without amino lipid. This lack of fusogenic activity probably results not only from the relatively high pK_a of **AL2**, which remains half-charged at this pH, but also from the small surface area occupied by the single hydrocarbon chain relative to the amino lipids with two chains. Bilayer destabilization requires a substantial imbalance between the size of the polar headgroup of the fusogenic lipid and its hydrophobic moiety. Esterification of the 2-position with acetate (**AL3**) produces a substantial increase in the fusion rate, and lengthening this second chain to butyryl (**AL4**) or decanoyl (**AL5**) again gives small increases, with rates approaching that achieved with **AL1**. Compound **AL6** with two decanoyl chains gave only limited lipid mixing, despite its comparably low pK_a. Clearly, the fusion induced by these compounds relies not only on the formation of the neutral form but also on their hydrocarbon structure.

DISCUSSION

Previous results from this laboratory have demonstrated that Ca^{2+}-induced fusion between LUVs can be regulated by the transbilayer distribution of anionic phospholipids such as PA (Eastman et al., 1992). The results presented here show that the regulatory effects of lipid asymmetry on fusion can be extended to LUV systems containing synthetic amino lipids in the absence of Ca^{2+} or other fusion-stimulating agents. Three points of interest are the ability of pH gradients to induce the asymmetry of amino lipids, the mechanism of fusion that results upon relaxation of this asymmetry, and the practical utility of this behavior.

For anionic lipids such as PA, only the protonated, neutral form is able to move across the bilayer (Eastman et al., 1991). In LUVs with a pH gradient, net transport then proceeds from the low-pH side of the bilayer to the high-pH side. In the case of amino lipids such as **AL1**, the deprotonated form is neutral and membrane-permeable, resulting in net transbilayer transport from high pH to low pH. As the pK_a of an amino function near the bilayer surface can be 7 or lower, net inward transport to an acidic interior can be achieved at physiological external pH values. The inward transport of **AL1** and the other amino lipids studied is extremely rapid at pH 7.5 and 20 °C. This is in contrast to the behavior of PA and other phospholipids, which require elevated temperatures and low external pH values for rapid transbilayer movement. The rapid transport of the amino lipids **AL1**–**AL6** can be attributed to the lack of the large, polar phosphate group of the phospholipids and to their pK_a's, which are such that an appreciable fraction of the neutral, membrane-permeable form is present at pH 7.5.

With regard to the mechanism of amino lipid-induced fusion, the behavior of **AL1**, when present in the outer monolayer of LUVs at pH values above the amino lipid pK_a, is consistent with the highly fusogenic character of diacylglycerols (Das & Rand, 1986), owing to the propensity of these compounds to induce non-bilayer structures, such as the hexagonal H_{II} phase. This is supported in the NMR data by the appearance of an H_{II} component in the EPC/DOPE/Chol/**AL1** multilamellar system at pH 7.5, which is not observed at pH 4.0. Thus, the results presented here are also consistent with a fusion process that relies on the ability of component lipids to adopt transitory non-bilayer structures such as inverted micelles (Cullis & Hope, 1978), interlamellar attachment sites (Siegel et al., 1989), or stalks (Siegel, 1993).

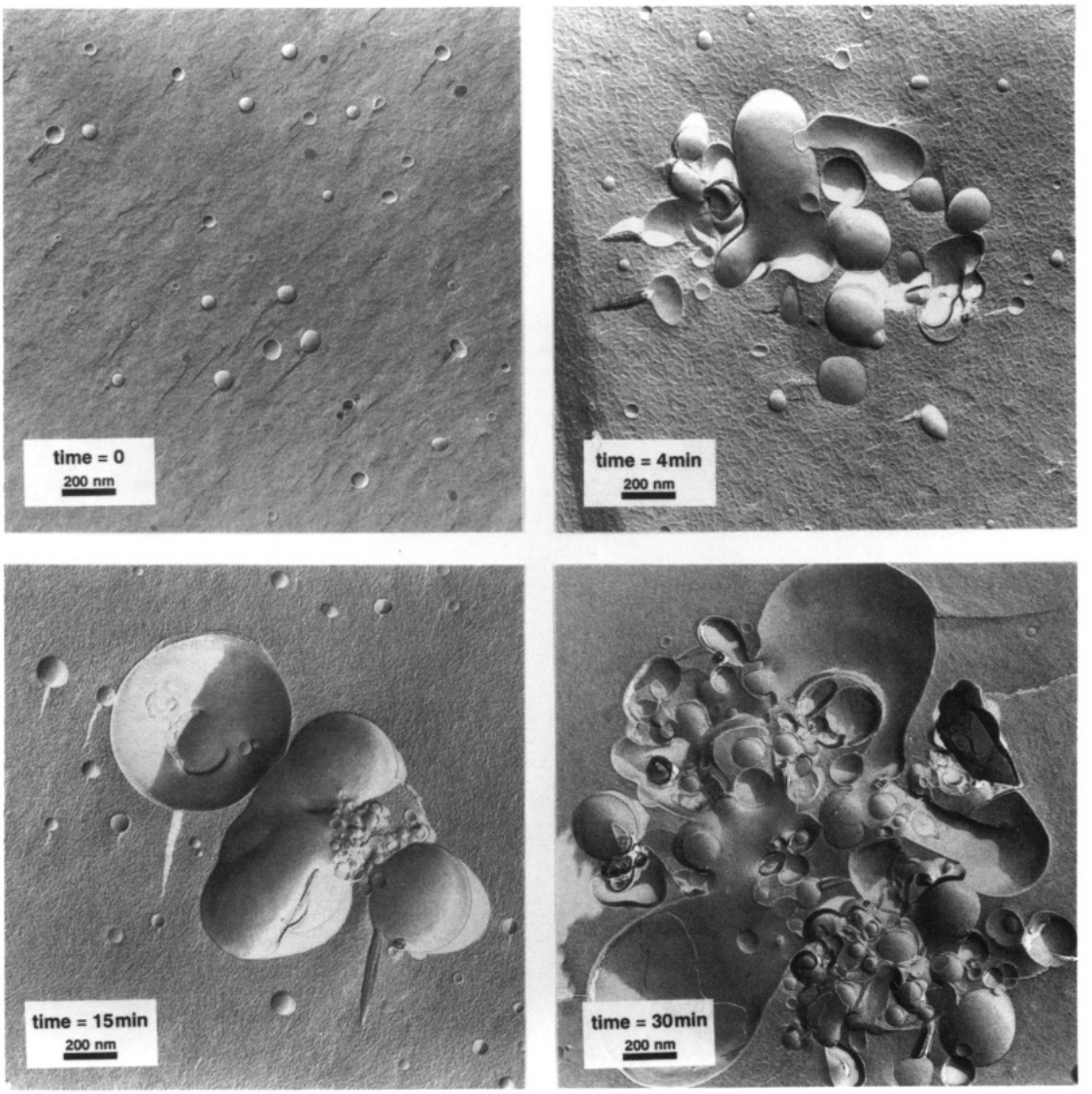

FIGURE 9: Freeze-fracture electron micrographs of 5 mol % **AL1** in EPC/DOPE/Chol (35:20:45), with a pH gradient (time = 0; internal, 300 mM sodium citrate (pH 4.0); external, 20 mM HEPES and 150 mM NaCl (pH 7.5)) and 4, 15, and 30 min after dissipation of the gradient by the addition of ammonium acetate to a final concentration of 100 mM. Lipid concentration was approximately 10 mM, and original magnification was 20 000×.

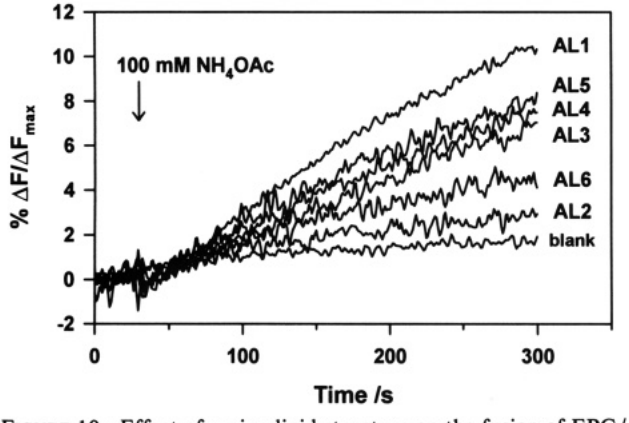

FIGURE 10: Effect of amino lipid structure on the fusion of EPC/DOPE/Chol (35:20:45) vesicles by RET fluorescent probe dilution. Liposomes were prepared with 5 mol % of the indicated amino lipid (**AL1**–**AL6**), an internal buffer of 300 mM sodium citrate (pH 4.0), and an external buffer of 20 mM HEPES and 150 mM NaCl (pH 7.5). Fusion assays were carried out as described in Materials and Methods.

The rate of membrane fusion has been shown to depend on the hydrocarbon structure of the amino lipid as well as its pK_a. Amino lipids **AL2**–**AL6** gave rates of fusion dependent upon the length and number of hydrocarbon chains in their structures. Compounds with longer hydrocarbon chains gave higher rates of fusion, even when the pK_a remained relatively constant. None of these compounds matched the fusion rate observed with **AL1**, a result that highlights the importance of long, unsaturated chains to membrane destabilization.

The ability to control the transbilayer asymmetry of the amino lipids described in this work using pH gradients, and to thereby control membrane fusion, has a number of potential applications. For example, the ability of liposomes to deliver encapsulated materials, such as DNA, to the interior of target cells requires fusion with the plasma membrane or, following endocytosis, the endosomal membrane. While cationic liposomes are now widely used for highly efficient nucleic acid transfections of mammalian cells *in vitro* (Malone et al., 1989), extension of this technology to *in vivo*-targeted delivery of liposomally encapsulated drugs and gene therapy has encountered problems of rapid clearance of liposomes, cellular toxicity, hemolysis, and accelerated clotting responses caused by the cationic lipids (Senior et al., 1991). These problems appear to occur above a threshold plasma concentration of positive charge and are not largely dependent on the nature of the cationic species.

The amino lipids described in this work may offer considerable advantages over cationic fusogenic lipids bearing permanent positive charges. Farhood et al. (1992) have shown that lipids bearing tertiary amines have lower cytotoxicity than quaternary amine analogues. Here we show that the low pK_a's of **AL1** and related compounds permit them to be maintained on the inner surface of the acidic vesicles with an exterior physiological pH. This may result in longer circulation residence times by reducing plasma protein interactions, which lead to clearance by the macrophages of the reticuloendothelial system.

In addition, the mechanism of cell entry for the system described here would be different from that proposed for cationic liposomes, which require high concentrations of polyanions (e.g., DNA) or oppositely charged membranes to give fusion (Düzgüneş et al., 1989; Stamatatos et al., 1988). **AL1** is capable of destabilizing a lipid membrane and causing fusion when present on the outer monolayer at physiological pH. In combination with liposomal targeting methods and passive gradient dissipation, fusion of liposomes with target plasma membranes might be achieved.

In summary, we have demonstrated the modulation of membrane fusion via pH gradient-induced asymmetric distributions of synthetic amino lipids. The protonatable lipids are charged and bilayer-stable at pH 4.0 when incorporated at 5 mol % into liposomes consisting of EPC/DOPE/Chol (35:20:45). The amino lipids can be rapidly sequestered to the inner monolayer of LUVs by increasing the external pH to 7.5 with retention of membrane stability. Dissipation of the pH gradient results in neutralization of the amino headgroups and redistribution of the amino lipids to the outer monolayer. In the neutral form, the amino lipids destabilize the membrane and cause membrane fusion by a process consistent with the formation of non-bilayer lipid intermediates.

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