pH-Dependent Stability and Fusion of Liposomes Combining Protonatable Double-Chain Amphiphiles with Phosphatidylethanolamine†

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Received October 8, 1986; Revised Manuscript Received January 9, 1987

ABSTRACT: We have prepared a series of novel double-chain amphiphiles with protonatable head groups, including acylated derivatives of various 2-substituted palmitic acids, amino acid conjugates of these species, and 1,2-dioleoyl-3-succinylglycerol. These species can be combined with phosphatidylethanolamine (PE) to prepare reverse-phase evaporation vesicles that are stable and trap hydrophilic solutes at pH 7. At weakly acidic pH values (as high as 6.5, depending on the titratable amphiphilic component), these pH-sensitive vesicles exhibit fusion, with a limited extent of contents mixing and extensive mixing of lipids, accompanied by leakage of aqueous contents. Protons and divalent cations show strong synergistic effects in promoting mixing of both lipids and aqueous contents between pH-sensitive vesicles prepared with any of a variety of double-chain titratable amphiphiles. Calorimetric results indicate that the relative stabilities of different types of pH-sensitive liposomes at low pH cannot be simply correlated with the propensity of the lipids to form a hexagonal II phase under these conditions. Fluorescence measurements demonstrate that single-chain fatty acids, but not double-chain titratable amphiphiles such as *N*-acyl-2-aminopalmitic acids, are rapidly removed from pH-sensitive vesicles in the presence of other lipid vesicles, serum albumin, or serum. Additionally, pH-sensitive liposomes containing double-chain titratable amphiphiles retain their aqueous contents better than do those containing single-chain amphiphiles in the presence of lipid membranes or albumin. Surprisingly, however, pH-sensitive vesicles of either type show retention of contents in the presence of serum that is comparable to that observed with vesicles composed purely of phospholipids. A model is proposed to explain these latter findings.

pH-sensitive liposomes, which form stable, sealed structures at neutral pH but become destabilized at weakly acidic pH, have attracted considerable interest in recent years for their potential to release encapsulated hydrophilic molecules at specific loci in cells or in organisms where a low local pH prevails. Vesicles of this type were first proposed and designed to afford enhanced release of contents in regions of the body where the local pH is reduced below normal due to pathological conditions such as inflammation, infection, or malignant transformation (Yatvin et al., 1980, 1983; Nayar & Schroit, 1985; Tegmo-Larsson et al., 1985). Recently, pH-sensitive liposomes have also been proposed for use to deliver encapsulated molecules to the cytoplasm of animal cells. A suitably constructed liposome, once endocytosed by a cell, can in principle be triggered to fuse with the membrane of an endocytic vesicle under the mildly acidic conditions prevailing in such vesicles, thereby releasing the liposomal contents to the cell cytoplasm (Connor et al., 1984; Straubinger et al., 1985a,b; Düzgünes et al., 1985; Connor & Huang, 1985).

To date, several combinations of lipids have been used to construct pH-sensitive liposomes that exhibit at least some of the properties discussed above, including in some cases the ability to promote the introduction of encapsulated hydrophilic molecules into mammalian cells (Straubinger et al., 1985a,b; Connor & Huang, 1985; Collins & Huang, 1986). Some of the most promising systems of this type are generated by using mixtures of a neutral phospholipid and a protonatable single-chain amphiphile, such as a fatty acid or an *N*-acylamino acid (Connor et al., 1984; Düzgünes et al., 1985). Single-chain amphiphiles of this type typically can dissociate readily from membranes in which they are incorporated (Doody et al., 1980; Brecher et al., 1984; Daniels et al., 1985; Hamilton & Cistola, 1986; Storch & Kleinfeld, 1986). This fact raises interesting and important questions regarding the stability of vesicles containing such amphiphiles in the presence of agents to which the amphiphiles can bind, and concerning the nature and mechanism(s) of the interactions between membranes and pH-sensitive liposomes. In the light of these considerations, we have prepared a series of double-chain amphiphiles that, like the single-chain amphiphiles noted above, carry a net negative charge at pH ≥7 but become protonated at mildly acidic pH. In this paper, we describe some important properties of lipid vesicles combining these double-chain amphiphiles with phosphatidylethanolamine (PE), including the pH-dependent destabilization and fusion of these vesicles and the stability of the vesicles in the presence of potential destabilizing agents, such as other membranes, serum albumin, and whole serum.

Materials and Methods

Materials

Egg yolk phosphatidylcholine and DOPC[1] were obtained from Avanti Polar Lipids (Birmingham, Al). TPE, POPE,

† This work was supported by the Medical Research Council of Canada (Grants MA-7776 and ME-75800) and les Fonds pour la Formation de Chercheurs et l'Aide à la Recherche du Québec (Grant 86-EQ-2954).

[1] Abbreviations: ANTS, 1-amino-3,6,8-naphthalenetrisulfonic acid disodium salt; *t*-Boc, *tert*-butyloxycarbonyl; 12-CPT-stearic acid; 12-[[[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]thioureidyl]stearic acid; (12-DABS)-18 PC, 1-palmitoyl-2-[12-[[[4-[[4-(dimethylamino)-phenyl]azo]phenyl]sulfonyl]methylamino]stearoyl]-*sn*-glycero-3-phosphocholine; DE, dielaidoyl; DO, dioleoyl; DPX, *N,N'-p*-xylylene-bis(pyridinium bromide); EDTA, ethylenediaminetetraacetic acid trisodium salt; LUV, large unilamellar vesicles; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; NBD-chloride, 4-chloro-7-nitrobenzofurazan; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, *sn*-glycero-3-phosphocholine; PE, *sn*-glycero-3-phosphoethanolamine; PG, *sn*-glycerol 3-phosphate; PO, 1-palmitoyl-2-oleoyl; Rho-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; Tes, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; TLC, thin-layer chromatography; TPE, phosphatidylethanolamine prepared from egg yolk phosphatidylcholine by transphosphatidylation.

0006-2960/87/0426-3267$01.50/0

and DOPG were prepared from egg yolk PC, POPC, and DOPC, respectively, by enzymatic transphosphatidylation as described previously (Comfurius & Zwaal, 1977), with the modification that the reactions catalyzed by phospholipase D were carried out at pH 6.8. Phospholipids were purified by silicic acid column chromatography and acetone precipitation as described previously (Silvius & Gagné, 1984). NBD-PE was obtained from Avanti, and Rho-PE was prepared from TPE as described previously (Struck et al., 1981). (12-DABS)-18 PC was synthesized as described elsewhere (Silvius et al., 1987). ANTS and DPX were obtained from Molecular Probes (Junction City, OR). Calcein (Sigma) was purified by chromatography on Sephadex LH-20 in distilled water by using a procedure similar to that described by Ralston et al. (1981) for the purification of carboxyfluorescein. Fetal bovine serum was obtained from Gibco Laboratories (Grand Island, NY) and serum albumin (fraction V) from Sigma.

N-Oleoyl- and N-Elaidoyl-2-aminopalmitic Acids (**1a** *and* **4a**). 2-Bromopalmitic acid (2 mmol) was reacted overnight with 10 mL of 33% aqueous NH_4OH in a sealed tube at 60 °C, and the reaction mixture was then cautiously acidified with concentrated HCl and filtered. The residue was washed with small amounts of distilled water and 1:1 methanol/water and then dried thoroughly in vacuo over P_2O_5. The crude 2-aminopalmitic acid (350 mg, 1.29 mmol) was esterified with methanol/HCl, isolated by partitioning between $CHCl_3$ and 1% aqueous Na_2CO_3, pH 10, and thoroughly dried in vacuo. The dried amino acid ester was reacted overnight at 20 °C with 1.3 and 2.5 molar equiv of acyl chloride and dry triethylamine, respectively, in dry CH_2Cl_2. The products were partitioned between 2:1 hexane/$CHCl_3$ and 1:1 methanol/0.1 N aqueous NaOH, the organic phase was concentrated in vacuo, and the residue was chromatographed on a column of Bio-Sil A (200–400 mesh), eluting with a gradient of diethyl ether in hexane to yield the pure methyl esters of **1a** and **4a** at 15% ether. Saponification with 0.5 M KOH (50 °C, 1 h) liberated the free acids **1a** and **4a**, which were normally recovered in pure form. When necessary, the product acids were freed of small amounts of impurities by chromatography on silicic acid, eluting with a gradient of 0–3% methanol in chloroform. Typical final yields were 40–50% on the basis of the starting bromo acid.

N-Oleoyl-2-(methylamino)palmitic acid (**3a**) was also synthesized by the above reaction sequence, by using 40% aqueous methylamine in place of ammonia in the first reaction and omitting the precipitation of the 2-(methylamino)palmitic acid. Instead, the reaction mixture containing the crude methylamino acid was repeatedly mixed with ethanol and evaporated in vacuo prior to esterification with methanolic HCl.

O-Oleoyl-2-hydroxypalmitic Acid (**2a**). 2-Bromopalmitic acid was converted to 2-hydroxypalmitic acid as described previously (Guest, 1947). Direct acylation of the hydroxy acid proved unsatisfactory for preparation of **2a**, as free fatty acid byproducts are difficult to resolve chromatographically from **2a**. Therefore, the hydroxy acid was esterified with diphenyldiazomethane (Adamson et al., 1975), and the benzhydrol ester product was chromatographed on silicic acid, eluting with 0–5% diethyl ether in hexane to yield pure hydroxy ester in 75% yield. The protected ester (500 mg, 1.14 mmol) was reacted for 40 h at 20 °C with 1.5 equiv of oleoyl chloride and 3 equiv of pyridine in dry ether and then partitioned between 2:1 hexane/$CHCl_3$ and 1:1 methanol/0.1 N aqueous NaOH. The organic phase was concentrated and the products were chromatographed on a silicic acid column, eluting with 0.5%, 0.75%, and 1% diethyl ether in hexane to yield the O-acylated ester at 1% ether. After cleavage of the protecting group with HCl in dry $CHCl_3$ (Silvius & Gagné, 1984), the deprotected product was purified on a column of silicic acid, eluting with 0–5% diethyl ether in hexane to yield pure **2a** (334 mg, 55% yield on the basis of protected 2-hydroxypalmitic acid) at 4% ether.

Synthesis of Amino Acid Conjugates. Species **1a**, **2a**, and **3a** were converted to *N*-hydroxysuccinimidyl esters, which were then used to prepare the L-serine and glycine conjugates **1b**, **1c**, **2b**, and **3b** in yields of ~80% (Lapidot et al., 1967). To prepare the histidine conjugate **1d**, the *N*-hydroxysuccinimidyl ester of **1a** was reacted with a fivefold excess of histidine methyl ester and dry triethylamine in dry CH_2Cl_2 for 16 h at 20 °C. After workup, the reaction products were applied in 1% acetic acid in $CHCl_3$ to a silicic acid column, which was eluted with 99:1 $CHCl_3$/methanol to remove contaminants and then with 98:2:0.25 $CHCl_3$/methanol/triethylamine to yield the pure methyl ester of **1d**. Cleavage with 0.5 M methanolic KOH for 1 h at 50 °C gave pure **1d** in an overall yield of 85% on the basis of **1a**.

1,2-Dioleoyl-3-succinylglycerol (*Succinyldiolein*, **5**). 1,2-Diolein (50 mg, 80 mmol) was dissolved in 3 mL of dry CH_2Cl_2 and reacted overnight with 16 mg of succinic anhydride and 11.9 mg of 4-pyrrolidinopyridine. The reaction was stirred overnight at 20 °C, and after the products were partitioned between hexane and 1:4 methanol/100 mM aqueous HCOONa, pH 3.0, they were chromatographed on a column of silicic acid, eluting with a gradient of 0–20% diethyl ether in hexane to yield pure species **5** at 20% ether.

All of the pH-sensitive lipid analogues whose syntheses are described above were digested extensively with 2 N KOH in aqueous ethanol at 75 °C and then esterified with methanol/HCl and analyzed by TLC, along with appropriate standards. In all cases, it was confirmed that the expected products were formed from these procedures.

Fluorescent Fatty Acids and Analogues of **1a**. 12-(Methylamino)stearic acid, its *N*-*t*-Boc derivative, and *N*-NBD-12-(methylamino)stearic acid were synthesized as described elsewhere (Silvius et al., 1987). 12-CPT-stearic acid was synthesized by reacting 12-(methylamino)stearic acid with 7-(diethylamino)-3-(4-isothiocyanatophenyl)-4-methylcoumarin and dry triethylamine (1.2 and 2 molar equiv, respectively) in dry CH_2Cl_2 for 12 h at 25 °C, and the product was purified by preparative TLC. The *N*-*t*-Boc derivative of 12-(methylamino)stearic acid was converted to the corresponding anhydride (Selinger & Lapidot, 1966) and coupled to methyl 2-aminopalmitate as described above for the synthesis of **1a**. The coupled product was purified by column chromatography and then treated successively with dry HCl in $CHCl_3$ at 0 °C for 2 h, to remove the *t*-Boc protecting group, and with 1 M KOH in methanol for 1 h at 50 °C to cleave the methyl ester function. The product, *N*-[12-(methylamino)stearoyl]-2-aminopalmitic acid, was labeled with a twofold excess of NBD-chloride and dry triethylamine in CH_2Cl_2 for 40 h at 25 °C, or with 7-(diethylamino)-3-(4-isothiocyanatophenyl)-4-methylcoumarin as described above for labeling of 12-(methylamino)stearic acid with this reagent. The fluorescent analogues of **1a** were purified by preparative TLC. All of the above reactions were carried out under N_2 and with exclusion of light.

Methods

Preparation of Liposomes. Large unilamellar vesicles (LUV) were prepared from various combinations of phospholipids and pH-sensitive amphiphiles by reverse-phase

evaporation and filtration through 0.1-μm Nucleopore membrane filters, as described by Wilschut et al. (1980). Vesicles were routinely prepared in 150 mM NaCl, 5 mM histidine, 5 mM Tes, and 0.1 mM EDTA, pH 7.8, or in solutions of equivalent osmolarity and pH. Vesicle encapsulated volumes were measured by using carboxyfluorescein as a marker of the interior aqueous space as described by Wilschut et al. (1980). Lipid concentrations were determined by phosphorus assay as described elsewhere (Silvius & Gagné, 1984).

Fusion and Leakage Assays. Mixing of vesicle contents was measured by using the procedure of Ellens et al. (1985), encapsulating 50 mM ANTS into one population of vesicles and 90 mM DPX, a water-soluble quencher of ANTS fluorescence, into the other. Leakage of contents from vesicles at low pH was measured by using the related procedure described by Ellens et al. (1984), in which 25 mM ANTS and 45 mM DPX are initially coencapsulated in the vesicles. Vesicles of this type were also used to estimate the maximum fluorescence response, corresponding to 100% mixing, in the contents-mixing assay. Leakage of vesicle contents in the presence of phospholipid vesicles, serum albumin, and serum was measured by using the ANTS/DPX assay and also by using the calcein-release assay described by Allen and Cleland (1980), with the modification that 75 mM calcein rather than 200 mM calcein was encapsulated in the vesicles. The fluorescence signal corresponding to complete release of contents in these assays was determined by lysing the vesicles with 0.1% Triton X-100. In all assays of vesicle contents mixing and leakage, the encapsulated solutions were buffered with 5 mM histidine and 5 mM Tes to pH 7.8 and adjusted with NaCl to an osmolarity equivalent to that of 150 mM NaCl, 5 mM histidine, and 5 mM Tes (determined by direct osmometry). Lipid mixing between vesicles was monitored by using the procedure of Struck et al. (1981), with the modification that labeled vesicles containing 1.0 mol % NBD-PE and 0.35 mol % Rho-PE were incubated with a ninefold excess of unlabeled vesicles. The fluorescence signal representing complete mixing of lipids was established by using control liposomes colabeled with 0.1 mol % NBD-PE and 0.035 mol % Rho-PE. Except where otherwise noted, all assays of fusion and leakage were carried out by using a vesicle concentration of 30 μM lipid at 42 °C for DEPE-containing vesicles and at 37 °C for TPE-containing vesicles. In experiments where vesicle destabilization and fusion was initiated by acidification of the aqueous medium, the vesicles were initially incubated in 150 mM NaCl, 2 mM histidine, 2 mM Tes, and 0.1 mM EDTA, pH 7.8, and the pH was abruptly reduced by injecting a small volume of a concentrated solution of Mes or acetate to give the desired final pH (verified by direct measurement).

Calorimetric Measurements. Lipid samples for calorimetry (5 μmol total lipid) were dried thoroughly under high vacuum and then dispersed by prolonged vortexing at 45 °C in 150 mM NaCl, 2 mM histidine, 2 mM Tes, and 0.1 mM EDTA, pH 7.8. Concentrated solutions of Mes or acetate buffer were then added to give a buffer concentration of 20 mM and the desired final pH (verified by direct measurement). The samples were freeze–thawed 3 times and then incubated for 15 min at 40 °C and cooled to 2 °C at ≤0.3 °C/min. After overnight incubation at 2 °C, the samples were analyzed by high-sensitivity differential scanning calorimetry (scan rate 25 °C/h) as described in Silvius and Gagné (1984).

RESULTS

Basic Properties of pH-Sensitive Amphiphiles. The structures of the double-chain titratable amphiphiles that were prepared in this study are shown in Figure 1. In Table I are listed chromatographic mobilities in three different solvent systems for the various pH-sensitive lipid analogues, and important precursors, that were synthesized in this study. Most of the pH-sensitive amphiphiles gave single spots on TLC, but

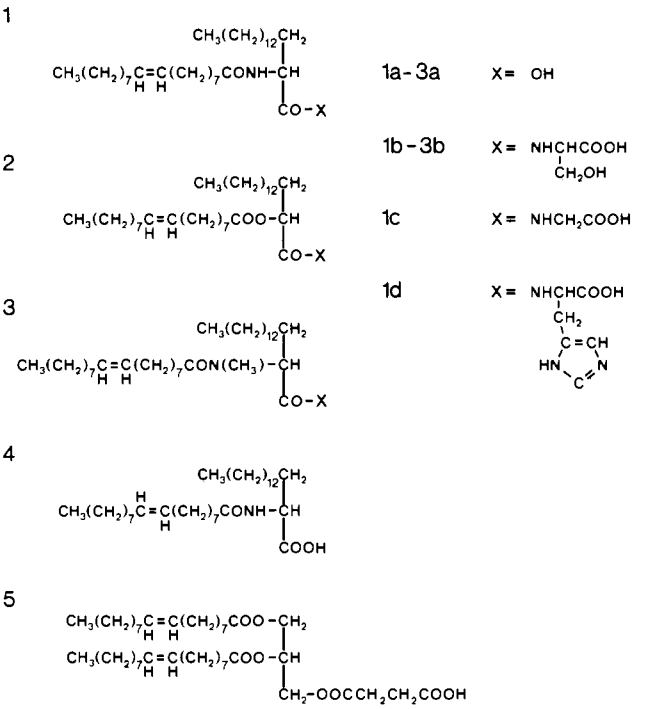

FIGURE 1: Structures of the titratable double-chain amphiphiles synthesized in this study.

Table I: Thin-Layer Chromatographic Mobilities of pH-Sensitive Lipid Analogues and Precursors

compound	R_f in solvent system[a] A	B	C
oleic acid	0.61	0.75	
2-hydroxyhexadecanoic acid	0.28	0.40	
2-hydroxyhexadecanoic acid benzhydrol ester	0.63	0.89	
2-(*cis*-9-octadecenoyloxy)hexadecanoic acid benzhydrol ester	0.91	~1.00	
2-(*cis*-9-octadecenoyloxy)hexadecanoic acid (**2a**)	0.52	0.64	
1,2-dioleoylglycerol	0.46	0.93	
1,2-dioleoyl-3-succinylglycerol (**5**)	0.38	0.76	
N-oleoyl-2-aminopalmitic acid methyl ester	0.29	0.87	
N-oleoyl-2-aminopalmitic acid (**1a**)	0.22	0.65	0.83
N-oleoyl-2-methylaminopalmitic acid (**3a**)	0.28	0.64	0.86
N-oleoylserine		0.32	0.60
N-(*N*-oleoyl-2-aminopalmitoyl)serine (**1b**)		0.46/0.58[b]	0.78
N-[*N*-oleoyl-2-(methylamino)-palmitoyl]serine (**3b**)		0.49	0.77
N-[2-(*cis*-9-octadecenoyloxy)hexadecanoyl]serine (**2b**)		0.49	0.79
N-(*N*-oleoyl-2-aminopalmitoyl)glycine (**1c**)		0.51	0.80
N^{α}-oleoylhistidine		0.05	0.20
N^{α}-(*N*-oleoyl-2-aminopalmitoyl)-histidine (**1d**)		0.06/0.11[b]	0.34/0.44[b]
N^{α}-(*N*-oleoyl-2-aminopalmitoyl)-histidine methyl ester		0.27[c]	0.55[c]

[a] Solvent systems: (A) 60:40:2 hexane/diethyl ether/acetic acid; (B) 90:10:1 $CHCl_3/CH_3OH$/acetic acid; (C) 50:15:5:5:2 $CHCl_3$/acetone/CH_3OH/acetic acid/H_2O. [b] Two spots of comparable intensity were observed, probably reflecting the presence of two diastereomers. [c] Two closely overlapping spots of comparable intensity were observed; an average R_f is given.

the L-serine and L-histidine conjugates **1b** and **1d** migrated as double spots of equal intensity. This chromatographic behavior appears to reflect the fact that the conjugates of racemic **1a** with L-serine and L-histidine constitute mixtures of diastereomers, which are (marginally) resolvable by chromatography. The L-serine conjugates **2b** and **3b** are also mixtures of diastereomers, but these do not appear to be resolvable by TLC.

With the single exception of the O-acylated 2-hydroxypalmitate species **2a**, all of the pH-sensitive lipid analogues studied here could be combined with DEPE, POPE, or TPE to generate stable dispersions of vesicles in good yield at pH 7.8. The stabilities and yields of vesicles prepared by the reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978; Wilschut et al., 1980) were generally optimal when the vesicles contained ~25 mol % of the pH-sensitive species together with PE. A few of the pH-sensitive amphiphiles, notably the serine conjugates **1b**, **2b**, and **3b**, gave stable vesicles when as little as 15 mol % of the amphiphile was combined with PE. In no case was the vesicle yield or stability markedly enhanced when the proportion of the pH-sensitive species in the vesicle was increased from 25 to 50 mol %. Interestingly, however, both species **1a** and its serine conjugate **1b** could form vesicles capable of trapping carboxyfluorescein at pH 7.8 even when no phospholipid was combined with these amphiphiles. In the remainder of this paper, except where we explicitly note otherwise, we will use the term "pH-sensitive liposomes (vesicles)" to denote specifically reverse-phase evaporation vesicles, composed of PE and a titratable lipid analogue in a 75:25 molar ratio (or a 70:30 molar ratio for PE/oleic acid vesicles), that have been prepared at pH 7.8 and passed through a 0.1-μm diameter Nucleopore membrane filter. Vesicles of this type typically exhibited trapped volumes in the range 2.0–4.0 μL/μmol of lipid, determined by carboxyfluorescein trapping as described by Wilschut et al. (1980).

Proton-Induced Fusion and Destabilization of Vesicles. Previous studies have demonstrated that, at weakly acidic pH, vesicles composed of PE and oleic acid can exhibit fusion, including a limited mixing of aqueous contents and substantial intermixing of lipids between vesicles, as well as considerable leakage of the vesicle contents (Düzgünes et al., 1985). We therefore examined the effects of pH on the fusion and destabilization of vesicles composed of dielaidoyl-PE combined with 25 mol % of the various pH-sensitive lipids described above or with 30 mol % of oleic acid.

In a first series of experiments, we examined the pH-dependent mixing of aqueous contents between various types of pH-sensitive vesicles, using the ANTS/DPX assay for contents mixing as described under Materials and Methods. Some representative results obtained in these experiments are shown in Figure 2. DEPE/oleic acid vesicles at 42 °C show contents mixing that is quite similar in its pH dependence, extent, and overall kinetics to the previously characterized fusion of egg PE/oleic acid vesicles at 25 °C (Düzgünes et al., 1985). A rapid but limited mixing of vesicle contents is observed when the pH is reduced from 7.8 to values ranging from roughly 4.5 to 6.5. The subsequent decay of the fluorescence signal arising from contents mixing indicates that this mixing of vesicle contents is followed by leakage of contents to the external medium.

Vesicles combining DEPE with species **1a** also exhibit pH-dependent mixing of contents, although the kinetics and the pH dependence of the contents mixing differ substantially from those observed for DEPE/oleic acid vesicles (Figure 2).

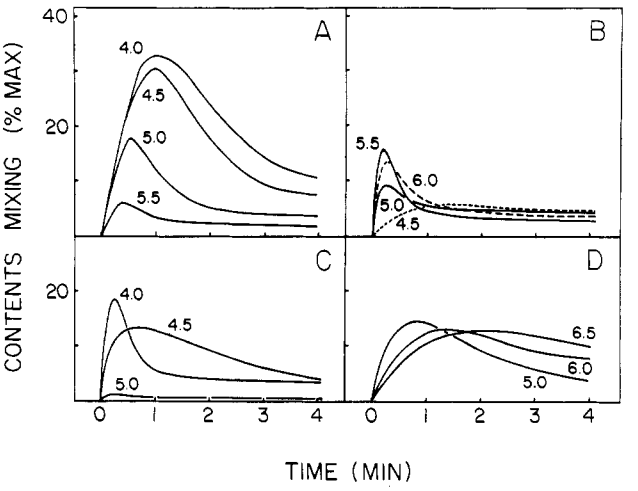

FIGURE 2: Time courses of contents mixing, monitored by the ANTS/DPX procedure of Ellens et al. (1985), for LUV combining DEPE with (A) 25 mol % species **1a**, (B) 30 mol % oleic acid, (C) 25 mol % species **1b**, or (D) 25 mol % species **1d**. At time zero, the pH of liposome suspensions (30 μM lipid) was reduced from 7.8 to the value indicated beside each curve. Other details of the assays were as described under Materials and Methods.

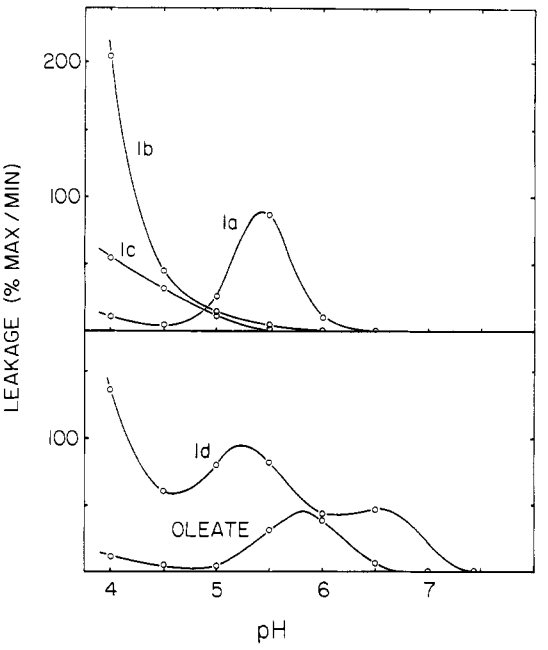

FIGURE 3: Initial rates of contents leakage (as percentage of contents released per minute) at varying pH for LUV combining DEPE with 25 mol % of various double-chain titratable amphiphiles (indicated beside each curve) or 30 mol % oleic acid. Leakage was assayed at 42 °C at a lipid concentration of 30 μM by the procedure of Ellens et al. (1984) as described under Materials and Methods.

Vesicles combining DEPE with the serine conjugate **1b** or with the histidine conjugate **1d** also show significant mixing of contents at weakly acidic pH, and again, each type of vesicle exhibits a distinctive pattern of dependence of the fusion kinetics on pH. Vesicles composed of DEPE together with most of the other pH-sensitive lipid analogues shown in Figure 1 typically showed less than 10% maximum contents mixing at pH 4.5 or above (results not shown).

The fluorescence time courses shown in Figure 2 indicate that leakage as well as mixing of vesicle contents takes place when pH-sensitive liposomes are exposed to acidic conditions. To examine this process of leakage more quantitatively, ANTS and DPX were coencapsulated in pH-sensitive liposomes at high concentrations, and leakage of vesicle contents was

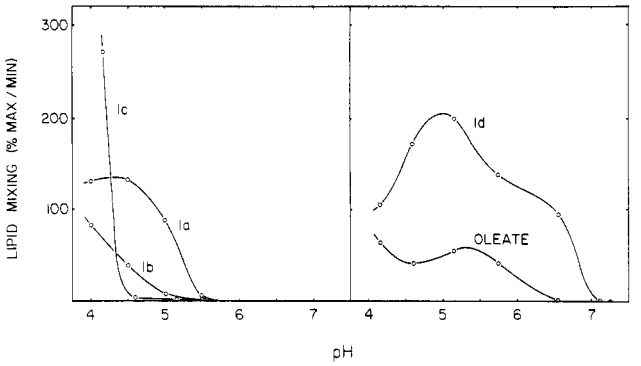

FIGURE 4: Initial rates of lipid mixing (as a percentage of maximal mixing per minute) at varying pH for LUV combining DEPE with 25 mol % of various double-chain titratable amphiphiles (indicated beside each curve) or 30 mol % oleic acid. Lipid mixing was assayed by the procedure of Struck et al. (1981), using 3 μM vesicles containing 1.0 mol % NBD-PE and 0.35 mol % Rho-PE, and 27 μM unlabeled vesicles. Other details were as described under Materials and Methods.

measured by the relief of quenching of ANTS fluorescence (Ellens et al., 1984). In Figure 3, we have plotted the initial rates of contents leakage from various types of pH-sensitive liposomes as a function of pH. All of these types of vesicles show negligible leakage at pH 7.4 and above. However, as the pH is reduced below neutral, each type of vesicle exhibits appreciable leakage over a range of pH values that follows fairly closely the range in which contents mixing is observed. For some types of pH-sensitive vesicles (most notably those containing DEPE and species **1a**), the rates of leakage measured at various pH values are not strictly correlated with the initial rates of contents mixing, although the pH thresholds for the two processes are comparable.

As an alternative method to monitor the coalescence of pH-sensitive vesicles under acidic conditions, we employed the lipid-mixing assay of Struck et al. (1981), which is not subject to the complicating effects of vesicle leakage. In Figure 4, the initial rates of lipid mixing for various types of pH-sensitive liposomes are plotted as a function of pH. For each type of liposome, the pH range in which lipid mixing is observed, as well as the relative rates of lipid mixing at different pH values, correlates well with the corresponding results obtained using the contents-mixing assay. The correlation between lipid mixing and leakage as a function of pH is weaker for some types of vesicles. While the two processes show similar pH thresholds for a given type of pH-sensitive liposome, the leakage rate typically reaches a maximum at a higher pH than does the lipid-mixing rate. This result may indicate that, for some types of pH-sensitive vesicles, the fusion process is more leaky at higher pH values. This conclusion must be viewed with caution, however, as we cannot exclude the possibility that vesicles prepared for the leakage assay, with high internal concentrations of ANTS and DPX, could be somewhat different in their stability properties from vesicles prepared for the lipid-mixing assay, which contain only buffer.

Calorimetric Studies. The results presented above indicate that the properties of pH-sensitive liposomes are strongly influenced by the nature of the titratable amphiphile that is combined with PE. This finding suggests that the pH-sensitive amphiphile in these liposomes not only provides a titratable charge but also modulates the physical properties of the lipid phase at low pH. To examine this possibility directly, we used differential scanning calorimetry to monitor the thermotropic properties of dispersions combining DEPE with various pH-sensitive lipid analogues at low pH. Representative thermograms obtained with such samples are shown in Figure 5, and

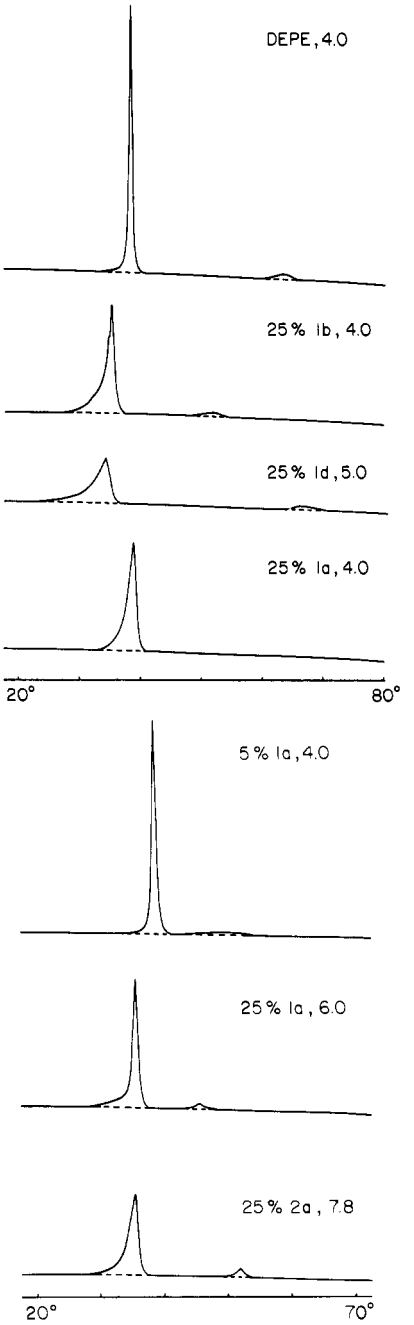

FIGURE 5: Calorimetric thermograms measured for dispersions of DEPE with the indicated molar percentages of species **1a**, **1b**, **1d**, or **2a**. The pH of each sample is also indicated next to each thermogram. Details of sample preparation and calorimetric analysis are described under Materials and Methods.

the results of a variety of these calorimetric experiments are summarized in Table II. Pure DEPE dispersed at pH 4.0 exhibits a large gel to liquid-crystalline transition at 38.2 °C, followed by a considerably smaller lamellar to hexagonal II transition centered at 63 °C, essentially the same temperature at which this transition takes place at pH 7.4 (Gagné et al., 1985; Epand, 1985). When various pH-sensitive amphiphiles, at levels up to 25 mol %, are combined with DEPE at weakly acidic pH, the main transition endotherm is typically broadened and shifted by at most a few degrees. By contrast, the temperature of the lamellar to hexagonal II transition endotherm (T_h) is shifted strongly downward by some titratable amphiphiles (e.g., species **1b** and particularly species **1a**) at low pH, while species **1d** slightly elevates T_h under similar conditions. By comparison of these results to those shown in

Table II: Main and Lamellar to Hexagonal II Phase Transition Temperatures for Mixtures of DEPE and Double-Chain Protonatable Amphiphiles[a]

sample	pH	T_c (°C)	T_h (°C)
DEPE	7.4	38.1	63.5
DEPE	4.0	38.2	63.0
DEPE/**1b** (75:25)	4.0	35.1	51.7
DEPE/**1b** (90:10)	4.0	36.9	58.1
DEPE/**1d** (75:25)	5.0	34.3	66.4
DEPE/**1d** (90:10)	5.0	36.3	64.6
DEPE/**1a** (75:25)	4.0	38.8	–[b]
DEPE/**1a** (90:10)	4.0	39.6	–[b]
DEPE/**1a** (85:5)	4.0	38.7	51.0
DEPE/**1a** (75:25)	5.0	38.3	–[b]
DEPE/**1a** (75:25)	6.0	35.4	45.5
DEPE/**1a** (75:25)	7.4	32.9	~70
DEPE/**2a** (75:25)	7.8	35.4	51.8

[a] T_c and T_h values were determined by high-sensitivity differential scanning calorimetry. Details of sample preparation and calorimetric analysis are described under Materials and Methods. [b] No separate high-temperature endotherm was observed by calorimetry. By varying systematically the pH and mole fraction of species **1a** in similar samples, we have concluded that the major transition in these samples represents a direct gel to hexagonal II transition.

Figures 2–4, it is clear that the fusion-promoting activity of a protonatable amphiphile cannot be correlated directly with its ability to promote the formation of a nonlamellar phase of PE at low pH.

Interestingly, 75:25 mixtures of DEPE with species **2a**, which replaces the amide-linked oleoyl chain of **1a** with an ester-linked oleoyl residue, exhibit a small but well-defined endotherm at 52 °C, in addition to a main transition centered at 35.4 °C, at pH 7.8. This observation suggests that liposomes composed of this lipid mixture exist near a lamellar to nonlamellar phase coexistence region even under the normal conditions we used to prepare pH-sensitive liposomes (37–40 °C, pH 7.8). This finding may account for our inability to obtain consistently well-dispersed preparations of vesicles by using this lipid mixture (see above). By contrast, all of the other titratable amphiphiles examined in this study elevated the T_h value for DEPE at pH 7.8 (results not shown).

Synergistic Effects of H^+ and Divalent Cations. Most of the types of pH-sensitive liposomes discussed above exhibit fusion, observable both by lipid-mixing and contents-mixing assays, in the presence of high concentrations of Ca^{2+} or Mg^{2+} (5–10 mM) at neutral pH (results not shown). Of greater interest here is the observation that lower concentrations of these divalent cations can act synergistically with protons to promote fusion of these vesicles at weakly acidic pH. Two examples of this phenomenon are shown in Figure 6 (upper panel), where the lipid-mixing assay has been used to monitor the coalescence of liposomes containing DEPE plus either species **1a** or the serine conjugate **1b**. At pH 5.5, vesicles containing DEPE and species **1a** in a 75:25 molar ratio show very slow lipid mixing. At pH 7.8, the addition of 2 mM $CaCl_2$ to the vesicles produces only a slow and limited mixing of lipids. However, the combination of pH 5.5 and 2 mM $CaCl_2$ causes much more rapid lipid mixing between vesicles than does either factor alone. An even more dramatic example of this synergy is observed in the case of vesicles combining 75 mol % DEPE with 25 mol % of the serine conjugate **1b**. These vesicles exhibit essentially no lipid mixing at pH 5.5, and addition of 2 mM Mg^{2+} or Ca^{2+} induces no mixing or a very slow mixing of lipids, respectively, at pH 7.8. However, the combination of a pH of 5.5 and 2 mM Ca^{2+} or Mg^{2+} produces a rapid and efficient mixing of lipids between vesicles.

Divalent cations can act synergistically with protons in promoting mixing of contents as well as lipids between pH-sensitive vesicles. Most noteworthy in this regard is the fact that low concentrations of divalent cations can enhance substantially the extent as well as the rate of proton-triggered contents mixing for some types of pH-sensitive vesicles. An example of such synergism between calcium and protons is observed in the fusion of vesicles containing PE and succinyldiolein (**5**) (Figure 6, lower panel). The addition of 2 mM Ca^{2+} at pH 7.8 produces virtually no mixing of vesicle contents, but a reduction in the pH to a value of 6.5 leads immediately to substantial mixing of contents, which is not observed at the lower pH in the absence of calcium. These and other examples of synergistic effects of protons and divalent cations on the fusion of pH-sensitive liposomes indicate that the efficiencies and the threshold pH values for fusion of these liposomes can be modified substantially by manipulating the concentrations of divalent cations in the aqueous medium.

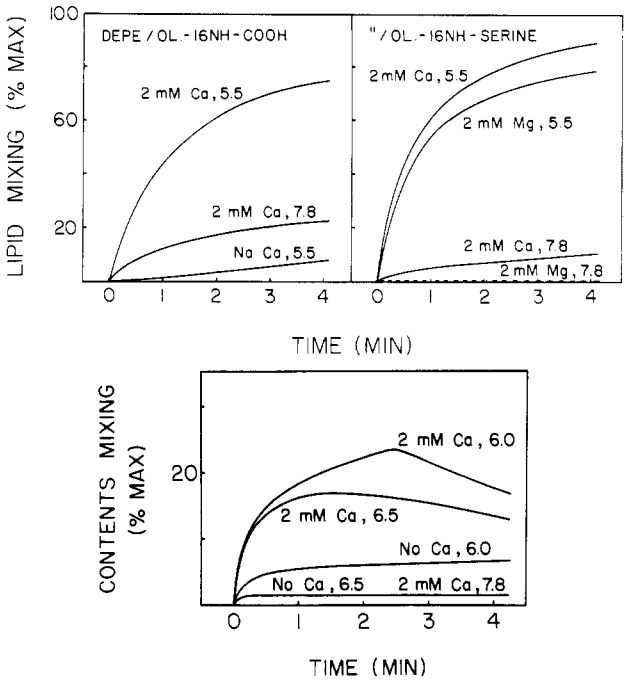

FIGURE 6: Upper panel: Time courses of lipid mixing, assayed as in Figure 5, for LUV composed of 75:25 DEPE/species **1a** (OL-16NH-COOH) or 75:25 DEPE/species **1b** (OL-16NH-SERINE). At time zero, calcium or magnesium chloride was added to 2 mM, at either pH 7.8 or pH 5.5 (as indicated), or the pH was abruptly lowered from 7.8 to 5.5 in the absence of divalent cations ("No Ca, 5.5" in left panel). Samples treated with divalent cations at pH 5.5 were first exposed to pH 5.5 for 30 s, causing negligible lipid mixing, before the injection of divalent cations. Vesicles containing DEPE and species **1b** gave no lipid mixing even after several minutes at pH 5.5 (not shown) when divalent cations were absent. Lower panel: Time courses of contents mixing, assayed as in Figure 2, after vesicles composed of 75:25 DEPE/species **5** (succinyldiolein) were exposed to 2 mM calcium chloride at different pH values. Calcium chloride was injected to 2 mM, where applicable, 10 s before the injection of Mes buffers to adjust the pH to 6.0 or 6.5, as indicated.

Effects of Lipid Membranes, Albumin, and Serum on Vesicle Stability. Single-chain amphiphiles such as fatty acids can exchange readily between membranes and other structures to which the amphiphiles can bind (Doody et al., 1980; Brecher et al., 1984; Daniels et al., 1985; Storch & Kleinfeld, 1986). Liposomes composed of PE together with such amphiphiles could rapidly become destabilized in the presence of membranes or amphiphile-binding molecules, as PE by itself does not form stable vesicles at neutral pH (Papahadjopoulos & Miller, 1967; Kolber & Haynes, 1979; Pryor et al., 1985). To examine this question, we carried out two types of experiments to examine the effects of lipid bilayer membranes, serum

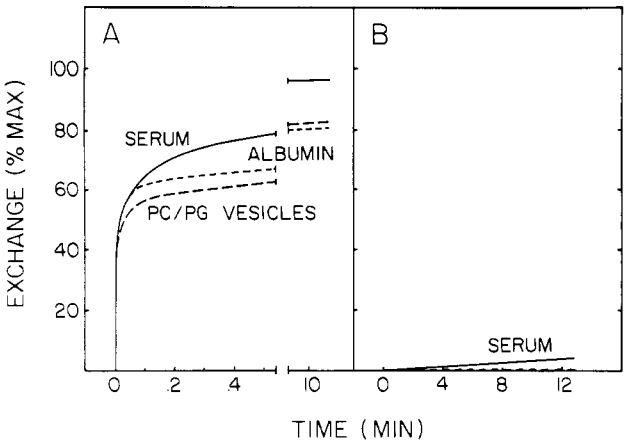

FIGURE 7: Time courses for dequenching of fluorescence, representing desorption of (A) *N*-NBD-12-(methylamino)stearic acid or (B) *N*-[*N*-NBD-12-(methylamino)stearoyl]-2-aminopalmitic acid, an analogue of species **1a**, from LUV prepared from 75 mol % TPE and 25 mol % fluorescent lipid. For the traces shown, either 25% fetal bovine serum, 10 μM serum albumin, or 60 μM sonicated POPC/DOPG vesicles was added at zero time to labeled vesicles (10 μM in the first two cases, 3 μM in the last). Similar results were obtained in (A) when 1% or 5% serum, 50 or 100 μM serum albumin, or 120 μM DOPC/DOPG vesicles was used in place of the concentrations of these agents specified above (not shown). In (B), no dequenching of fluorescence was seen up to 15 min with albumin or POPC/DOPG vesicles. Fluorescence values corresponding to complete equilibration of the labeled species throughout the system were determined by incubating samples for long times (several hours) with serum [or, in (A), with albumin] or by incorporating 1.12 nmol of either labeled probe into 180 nmol of POPC/DOPG vesicles in a final volume of 3 mL. For the case of albumin treatment of vesicles containing TPE and *N*-[*N*-NBD-12-(methylamino)stearoyl]-2-aminopalmitic acid, we assumed that the dequenching of fluorescence accompanying transfer of a probe molecule from the vesicles to albumin would be similar to the dequenching accompanying transfer of the probe from the vesicles to serum.

albumin, or whole serum on the stability of vesicles combining PE with either single-chain fatty acids or *N*-acyl-2-aminopalmitic acids (analogues of species **1a**).

In a first series of experiments, we measured the rate at which fluorescent-labeled fatty acids or *N*-acyl-2-aminopalmitic acids are extracted from pH-sensitive liposomes in the presence of phosholipid vesicles, serum albumin, or whole serum. Liposomes were prepared by using 75 mol % TPE together with 25 mol % of a fluorescent fatty acid, *N*-NBD-12-(methylamino)stearic acid, or with *N*-[*N*-NBD-12-(methylamino)stearoyl]-2-aminopalmitic acid, a fluorescent analogue of species **1a**. The fluorescence of the labeled species in these vesicles is strongly self-quenched, and desorption of the fluorescent molecules from the vesicles in the presence of membranes, serum, and other agents can readily be monitored as an enhancement of probe fluorescence (Nichols & Pagano, 1981; Silvius et al., 1987).

In Figure 7 are shown experimental results comparing the rates of desorption of *N*-NBD-12-(methylamino)stearic acid and the corresponding labeled *N*-acyl-2-aminopalmitic acid from pH-sensitive liposomes in the presence of various agents. The single-chain fluorescent fatty acid is removed very rapidly from PE/fatty acid vesicles when bovine serum albumin (10 μM), POPC/DOPG vesicles (20-fold excess), or as little as 1% fetal bovine serum is added. In each case, roughly half of the fatty acid is removed within at most a few seconds, while the balance of the fatty acid exchanges over a slower time course. The amplitude of the fast component was not significantly enhanced by further increasing the levels of the fatty acid acceptors (results not shown). We suggest that the fast component of exchange represents the desorption of fatty acid molecules that are initially present in the outer monolayers of the TPE/fatty acid vesicles. In contrast to the behavior of the labeled single-chain fatty acid, the labeled **1a** analogue *N*-[*N*-NBD-12-(methylamino)stearoyl]-2-aminopalmitic acid showed no detectable exchange from pH-sensitive vesicles over a time course of 15 min in the presence of excess POPC/DOPG vesicles or 10 μM serum albumin. In the presence of 25% fetal bovine serum, a slow dequenching of the probe fluorescence is seen, corresponding to an exchange rate of 0.39%/min. At a serum concentration of 5%, the apparent rate of probe exchange was reduced to 0.18%/min.

The experiments discussed above were carried out by using pH-sensitive vesicles containing high proportions of fluorescent probes. To ensure that the results obtained were not strongly influenced by the high levels of probes that were used, we employed a second approach to corroborate these findings. We have shown elsewhere (Silvius et al., 1987) that the fluorescence of lipids labeled with certain substituted coumarin moieties can be efficiently quenched by low bilayer concentrations of lipids labeled with a [[(dimethylamino)phenyl]-azo]benzene (DAB) group. We therefore prepared pH-sensitive vesicles from PE and either 30 mol % oleic acid or 25 mol % species **1a**, including in the former vesicles 0.5 mol % of a coumarin-labeled fatty acid, 12-CPT-stearic acid, and in the latter 0.5 mol % of the corresponding analogue of species **1a**, *N*-(12-CPT-stearoyl)-2-aminopalmitic acid, together with 2.5 mol % of a DAB-labeled phosphatidylcholine, (12-DABS)-18 PC. Measurements of the exchange of the coumarin-labeled probes from these vesicles in the presence of unlabeled vesicles, albumin, and serum, carried out again by monitoring the dequenching of probe fluorescence, gave results in good agreement with those obtained by using NBD-labeled lipids (data not shown). The coumarin-labeled fatty acid exchanged somewhat more slowly under all conditions than did its NBD-labeled counterpart, a difference that we have also noted previously in measurements of the exchange of phosphatidylcholines labeled with the two different fluorophores (Silvius et al., 1987). The coumarin-labeled *N*-acyl-2-aminopalmitic acid showed negligible exchange (<2%) over a time course of 15 min under all conditions.

As a second test of the stabiity of different pH-sensitive liposomes, we examined the release of trapped solutes from vesicles composed of TPE/oleic acid (70:30) or TPE/species **1a** (75:25) in the presence of PC/PG liposomes, albumin, or serum. Vesicle leakage was assayed by using the ANTS/DPX procedure of Ellens et al. (1984) as discussed above or the calcein-release assay described by Allen and Cleland (1980). In all cases, comparable results were obtained by the two methods. Both procedures were used to ensure the reliability of results obtained with high concentrations of serum, which contributed substantial background fluorescence in the ANTS/DPX assay and quenched partially the fluorescence of calcein.

In Figure 8A are shown fluorescence time courses, obtained by using the calcein-release assay, that illustrate the leakage of contents from TPE/oleic acid vesicles under various treatments. Initial rates of leakage measured in these and other experiments are summarized in Table III. The basal rate of leakage from TPE/oleic acid vesicles at pH 7.4 is negligible. In the presence of a tenfold excess of POPC/DOPG liposomes, substantial leakage is observed. The rate of leakage (as percent contents released per minute) increases almost linearly as the concentrations of TPE/oleic acid vesicles and PC/PG liposomes are increased in parallel. Leakage of contents from TPE/oleic acid vesicles can also be induced by

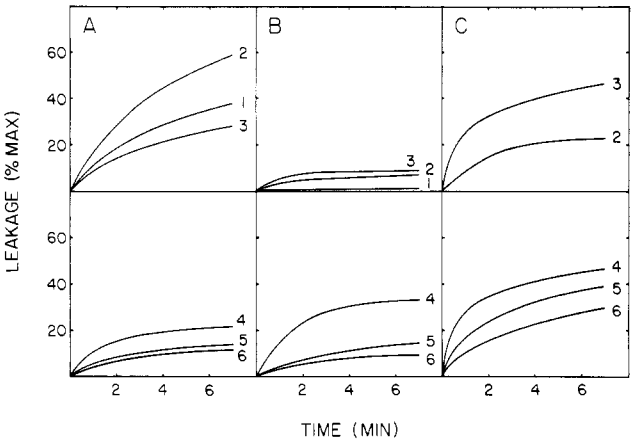

FIGURE 8: Time courses of leakage of calcein, monitored by relief of self-quenching as described under Materials and Methods, from LUV composed of (A) 70:30 TPE/oleic acid, (B) 75:25 TPE/species **1a**, or (C) 75:25 POPC/DOPG. At time zero, vesicles (10 μM) equilibrated at 37 °C with isotonic buffer at pH 7.8 were exposed to the following agents: curve 1, 100 μM sonicated POPC/DOPG vesicles; curve 2, 10 μM serum albumin; curve 3, 100 μM serum albumin; curves 4, 5, and 6, 25%, 5%, and 1% fetal bovine serum, respectively. Other experimental details are described in the text.

Table III: Effect of Vesicle Concentration on Calcein Leakage Induced from TPE/Oleic Acid Vesicles by Various Agents[a]

vesicle lipid concn (μM)	destabilizing agent	contents leakage rate (%/min)
3	PC/PG vesicles (30 μM)	4.8
3	PC/PG vesicles (100 μM)	11.5
10	PC/PG vesicles (100 μM)	13.8
10	PC/PG vesicles (300 μM)	22.9
1	albumin (10 μM)	4.4
3	albumin (10 μM)	11.8
10	albumin (10 μM)	28.2
10	serum (1%)	3.8
10	serum (25%)	7.0
30	serum (1%)	1.7
30	serum (25%)	4.8

[a] Calcein-loaded LUV were prepared from a 70:30 (mol/mol) mixture of TPE and oleic acid, and leakage was measured by relief of self-quenching of calcein fluorescence, as described under Materials and Methods. Assays were carried out at 37 °C.

serum albumin (10 μM), and again the rate of leakage varies directly with the vesicle concentration. The strong dependence of the leakage rate on the vesicle concentration in these experiments indicates that the release of contents from TPE/oleic acid vesicles under these conditions is mediated by interactions between vesicles. Interestingly, increasing the concentration of albumin from 10 to 100 μM substantially reduces the leakage rate (Figure 8A). We suggest that this rather surprising effect arises from a weak adsorption of albumin (or a contaminating protein) to the vesicles, which hinders direct contact between vesicle surfaces to a progressively greater extent as the amount of adsorbed protein increases.

Incubation of TPE/oleic acid vesicles with various volume percentages of fetal bovine serum caused leakage of vesicle contents that differed in two significant ways from the leakage induced by serum albumin. First, the rate of serum-induced leakage increased substantially as the concentration of serum was increased up to at least 25 vol %. Second, the leakage rate measured upon addition of serum was found to fall off rather than to increase as the concentration of calcein-loaded vesicles was increased. The rate of contents leakage in the presence of low concentrations of serum (1–5 vol %) is considerably less than that measured in the presence of an equivalent amount of serum albumin (which constitutes roughly 4% of the weight of whole serum).

The leakage experiments described above were also carried out with vesicles composed of TPE and species **1a**, which unlike oleic acid desorbs very slowly from the vesicle membrane (see above). Addition of a ten-fold excess of POPC/DOPG liposomes to these vesicles led to a very slow leakage of vesicle contents, the rate of which was not markedly dependent on the vesicle concentration (Figure 8B and Table III). When serum albumin was added to the vesicles, slightly faster initial rates of contents release were observed, but the rate of leakage rapidly fell to almost zero after only a small portion of the contents had been released (Figure 8B). These results indicate that vesicles composed of TPE and species **1a** are much more stable in the presence of PC/PG vesicles or serum albumin than are vesicles composed of TPE and oleic acid. By contrast, serum at concentrations from 1 to 25 vol % was found to induce leakage from TPE/**1a** vesicles at rates comparable to those observed with TPE/oleic acid vesicles at the same concentration of serum. Vesicles composed of POPC and DOPG (3:1) showed leakage in the presence of albumin and serum that was similar in rate and extent to that observed with TPE/**1a** vesicles under comparable conditions (Figure 8C). Therefore, vesicles that contain neither PE nor a protonatable amphiphile can still be significantly destabilized by albumin or serum. Interestingly, POPC/DOPG vesicles showed considerably greater leakage than did TPE/**1a** vesicles in the presence of high levels of serum (25%) or serum albumin (100 μM).

Discussion

The results presented in this study demonstrate that lipid vesicles composed of PE and titratable double-chain amphiphiles can exhibit pH-dependent fusion and destabilization that is similar in many ways to that reported previously for vesicles combining PE with other titratable amphiphiles (Connor et al., 1984; Ellens et al., 1984, 1985; Düzgünes et al., 1985; Straubinger et al., 1985b). In these respects, it does not appear that single-chain titratable amphiphiles confer unique properties on pH-sensitive liposomes that an appropriately chosen double-chain amphiphile, with similar pH sensitivity, cannot. However, each type of pH-sensitive liposome examined in this study (including PE/oleic acid vesicles) shows a distinctive pattern of pH dependence for destabilization and fusion, indicating that the precise structure of the titratable component in a pH-sensitive liposome is an important determinant of the liposomal properties at low pH.

The ability of PE to form nonlamellar phases under certain conditions has recently attracted considerable attention as a potential determinant of the stability and fusion of liposomes containing this lipid (Cullis & de Kruijff, 1979; Verkleij, 1984; Siegel, 1984, 1986; Ellens et al., 1984, 1985, 1986; Bentz et al., 1985). At least one titratable amphiphile, cholesterol hemisuccinate (CHEMS), has been shown to stabilize the lamellar phase of PE when negatively charged, while the protonated form of CHEMS has the opposite effect (Lai et al., 1985). However, while some of the protonatable analogues examined in the present study (e.g., species **1a** and **1b**) share this property with CHEMS, at least one titratable analogue studied here, species **1d**, does not. It is thus not clear that the efficiencies of fusion and destabilization of different types of pH-sensitive liposomes at low pH can be predicted by examining the physical properties of the component lipids in bulk dispersions.

In the light of the results obtained in this study, it might be asked which of the double-chain pH-sensitive amphiphiles generated here might be most useful for potential applications

in constructing pH-sensitive liposomes that can interact with animal cells. This question cannot be answered simply, however, as criteria other than the physical properties of the liposomes (e.g., cytotoxicity) may play an important role in selecting the type of pH-sensitive liposome to be used in a given application. Vesicles combining PE with species **1a** or its histidine conjugate **1d** become destabilized and can show significant fusion, including mixing of contents, at pH values likely to be encountered in endocytic vesicles [roughly 5.0–6.5 (Tycko & Maxfield, 1980; Tycko et al., 1983; Yamashiro et al., 1984)], even when divalent cations are wholly absent. Several other species, including the serine conjugates **1b**, **2b**, and **3b** and succinyldiolein (**5**), can show efficient fusion in this pH range if divalent cations are present at concentrations too low to elicit significant vesicle fusion at pH 7.4 (i.e., 1–2 mM). This fact may be of particular advantage since some of these latter titratable analogues, such as species **5**, which contains only ester as opposed to amide bonds, may be more readily metabolizable than are **1a** and its amino acid conjugates.

As discussed above, pH-sensitive liposomes constructed using double- vs. single-chain amphiphiles appear to show no fundamental and inherent differences in behavior at low pH. However, vesicles of the two types do differ substantially in the exchangeability of their titratable amphiphiles, and hence in the stability of the vesicles in the presence of agents that can bind such amphiphiles. Our measurements of the exchange of an NBD-labeled analogue of stearic acid indicate that this species desorbs rapidly (over a time course of the order of seconds) from the outer surfaces of PE/fatty acid vesicles in the presence of serum, serum albumin, or phospholipid vesicles. Other workers have previously reported comparably fast exchange of other long-chain fatty acids, such as 12-anthroylstearic acid and oleic acid, between different lipid bilayers or between lipid bilayers and albumin (Brecher et al., 1984; Daniels et al., 1985; Storch & Kleinfeld, 1986). This result is interesting because it suggests that PE/fatty acid vesicles, when incubated with cells, may rapidly become depleted of fatty acids as the fatty acid molecules desorb from the vesicles and partition into cell surface membranes or other hydrophobic compartments in the system. This loss of vesicle-associated fatty acids may not automatically lead to vesicle collapse, as the destabilization of PE-rich vesicles often requires direct contact with other lipid surfaces (Ellens et al., 1984) and can be effectively prevented when even relatively small amounts of protein are associated with the vesicle surface (Ho et al., 1986). In accord with these suggestions, we find that the kinetics of leakage of contents from TPE/oleic acid vesicles in the presence of serum albumin (or PC/PG vesicles) indicate that the leakage requires vesicle–vesicle interactions.

As we have noted under Results, pH-sensitive vesicles composed of TPE and a double-chain amphiphile, species **1a**, show much less leakage in the presence of PC/PG vesicles or serum albumin than do TPE/oleic acid vesicles. Our exchange experiments with fluorescent analogues of **1a** suggest that this difference is attributable to the fact that **1a** does not desorb significantly from pH-sensitive liposomes, at least on a time scale of several minutes, when albumin or other membranes are present. It is therefore at first sight rather surprising to find that TPE/oleic acid vesicles and TPE/**1a** vesicles show similar rates of leakage in the presence of various levels of serum. However, our results suggest that the leakage observed from these vesicles in the presence of serum is different in mechanism from the leakage observed in the presence of serum albumin or other lipid vesicles. First, the rate of leakage of contents from TPE/oleic acid vesicles at low concentrations of serum is considerably slower than that observed in the presence of an equivalent level of serum albumin (Table III). Second, the rate of leakage of contents from these vesicles increases as the level of serum is increased from 1% to 25% by volume, even though either concentration of serum can elicit a very rapid and extensive desorption of fatty acids from the vesicles. Finally, increasing the concentration of TPE/oleic acid (or TPE/**1a**) vesicles actually decreases the rate of leakage induced by serum, in contrast to the results observed when albumin or phospholipid vesicles were used to induce leakage. We suggest on the basis of these results that serum proteins adsorb to pH-sensitive vesicles, hindering direct contact between vesicles, and that leakage of contents from these vesicles in the presence of serum reflects a direct destabilizing action of serum components on individual, isolated vesicles. This proposal would explain our finding that serum at low levels causes much slower leakage of contents from TPE/oleic acid vesicles than does the equivalent amount of serum albumin, since serum albumin allows the interactions between fatty acid depleted vesicles that lead to rapid vesicle collapse. Our proposal also allows us to rationalize the fact that pH-sensitive liposomes containing either a rapidly exchangeable fatty acid or the double-chain amphiphile **1a** are destabilized at comparable rates by a given concentration of serum. The concentration of charged amphiphiles in the surface of a PE-rich vesicle may have less effect on the vesicles's interaction with serum components than on its interactions with other vesicles of like composition.

In summary, our results demonstrate that titratable double-chain amphiphiles can be combined with PE to construct pH-sensitive liposomes whose stability and physical properties largely resemble the corresponding properties of vesicles combining PE with titratable single-chain amphiphiles. However, vesicles containing double-chain amphiphiles such as species **1a** lose their titratable component much more slowly in the presence of serum albumin, serum, or lipid bilayers than do vesicles containing long-chain fatty acids. This difference is reflected in an enhanced retention of aqueous contents by pH-sensitive vesicles containing species **1a** rather than oleic acid under some conditions (e.g., in the presence of albumin or phospholipid bilayer membranes), although the two types of vesicles may show comparable retention of contents under others (as in the presence of serum). It will now be of interest to compare the interactions of cells with pH-sensitive liposomes that incorporate double- vs. single-chain amphiphiles.

Registry No. **1a**, 107743-20-4; **1a** methyl ester, 107768-23-0; **1a** *N*-hydroxysuccinimidyl ester, 107768-24-1; **1b** (isomer 1), 107743-31-7; **1b** (isomer 2), 107797-39-7; **1c**, 107743-32-8; **1d** (isomer 1), 107743-35-1; **1d** (isomer 2), 107797-42-2; **1d** methyl ester (isomer 1), 107743-36-2; **1d** methyl ester (isomer 2), 107797-43-3; **2a**, 107743-23-7; **2a** *N*-hydroxysuccinimidyl ester, 107743-29-3; **2b** (isomer 1), 107743-34-0; **2b** (isomer 2), 107797-41-1; **3a**, 107743-22-6; **3a** *N*-hydroxysuccinimidyl ester, 107743-30-6; **3b** (isomer 1), 107743-33-9; **3b** (isomer 2), 107797-40-0; **4a**, 107743-21-5; **4a** methyl ester, 107743-27-1; **5**, 107743-26-0; 12-CPT-stearic acid, 107768-25-2; NBD chloride, 10199-89-0; DEPE, 19805-18-6; oleic acid, 112-80-1; calcium, 7440-70-2; bromopalmitic acid, 65269-90-1; 2-aminopalmitic acid, 98320-69-5; *N*-elaidoyl chloride, 7459-35-0; 2-aminopalmitic acid methyl ester, 107743-28-2; 2-hydroxypalmitic acid, 764-67-0; 2-hydroxypalmitic acid benzhydrol ester, 107743-24-8; oleoyl chloride, 112-77-6; *O*-oleoyl-2-hydroxypalmitic acid benzhydrol ester, 107743-25-9; histidine methyl ester, 1499-46-3; L-serine, 56-45-1; glycine, 56-40-6; 1,2-diolein, 2442-61-7; succinic anhydride, 108-30-5; 12-(methylamino)stearic acid, 107743-38-4; 7-(diethylamino)-3-(4-isothiocyanatophenyl)-4-methylcoumarin, 107743-39-5; *N*-*t*-Boc-12-(methylamino)stearic acid anhydride, 107743-40-8; methyl 2-aminopalmitate, 107743-41-9; *N*-[12-(methylamino)stearoyl]-2-

aminopalmitic acid, 107743-43-1; *N*-[*N*-NBD-12-(methylamino)-stearoyl]-2-aminopalmitic acid, 107743-43-1; *N*-(12-CPT-stearoyl)-2-aminopalmitic acid, 107743-44-2; N^{α}-oleoylhistidine, 107768-12-7; *N*-oleoylserine, 107743-37-3.

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