N-Succinyldioleoylphosphatidylethanolamine: structural preferences in pure and mixed model membranes

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The structural preferences of the pH-sensitive phospholipid, N-succinyldioleoylphosphatidylethanolamine (N-succinyl-DOPE), have been examined alone and in mixtures with DOPE by ^{31}P-NMR, fluorescence energy transfer, and freeze-fracture techniques. The basic polymorphic behavior of pure N-succinyl-DOPE and DOPE/N-succinyl-DOPE lipid systems and the influence of calcium and pH were investigated. It is shown that, similar to other negatively charged acidic phospholipids, N-succinyl-DOPE adopts the bilayer organization upon hydration. This structure is maintained at both pH 7.4 and 4.0 in the presence or absence of calcium. In the mixed lipid system, N-succinyl-DOPE can stabilize the non-bilayer lipid, DOPE, into a bilayer structure at both pH 7.4 and 4.0 at more than 10 mol% N-succinyl-DOPE, although a narrow ^{31}P-NMR lineshape is observed at acidic pH values. This corresponds to the presence of smaller vesicles as shown by quasi-elastic light scattering measurements. Addition of equimolar calcium (with respect to N-succinyl-DOPE) to the DOPE/N-succinyl-DOPE systems induces the hexagonal H_{II} phase at both pH values. In unilamellar systems with similar lipid composition the addition of Ca^{2+} results in membrane fusion as indicated by fluorescence energy-transfer experiments. These findings are discussed with regard to the molecular mechanism of the bilayer to hexagonal H_{II} phase transition and membrane fusion and the utility of N-succinyl-DOPE containing pH-sensitive vesicles as drug-delivery vehicles.

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Abbreviation: DOPE, dioleoylphosphatidylethanolamine; N-Rho-PE, N(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; N-NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine. MLV, multilamellar vesicles; LUVETs, large unilamellar vesicles by extrusion techniques.

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Introduction

Acidic phospholipids can play important roles in a variety of membrane-mediated processes, including membrane fusion [1,2]. In addition, interactions between the negatively-charged acidic phospholipids and charged molecules and proteins can modulate membrane properties. For example, the gel-liquid crystalline transition of acidic phospholipids can be regulated by changes in pH or divalent cation concentration [3,4]. In the presence of calcium, phosphatidylserine and phosphatidic

acid can adopt anhydrous crystalline bilayers and segregate from other membrane lipids (lateral phase separation) [5–8]. Calcium can also induce drastic changes in the (liquid-crystalline) structural organization of lipids such as cardiolipin, where a lamellar to hexagonal H_{II} transition is induced [9,10]. Furthermore, divalent cations can cause fusion of membranes containing acidic phospholipids as can low pH in PS containing systems [11].

It has recently been shown that several synthetic acidic lipids such as cholesteryl hemisuccinate [12] and N-succinyl-DOPE [13] can be incorporated into phospholipid bilayers. Furthermore, these lipids can be employed to generate pH-sensitive liposomes when used in combination with phosphatidylethanolamine (PE). For example, it has been shown that pure N-succinyl-DOPE vesicles preferably leak their entrapped aqueous contents at neutral pH, whereas vesicles composed of N-succinyl-DOPE and DOPE are more leaky under acidic conditions [13]. The utility of these specialized lipids to generate pH-sensitive liposomes has been proposed for enhancing the therapeutic potential of liposome-based drug delivery systems under various physiological environments such as low pH and in efficient intracellular delivery of liposome-entrapped compounds via proton induced liposome-endosome membrane fusion.

In order to elucidate the mechanism of membrane destabilization in pure N-succinyl-DOPE and DOPE/N-succinyl-DOPE vesicles, the polymorphic phase preferences of N-succinyl-DOPE alone and DOPE/N-succinyl-DOPE mixtures have been investigated by ^{31}P-NMR, freeze-fracture, and fluorescence energy-transfer techniques. Our results indicate that the polymorphic behaviour of N-succinyl-DOPE is similar to that of other acidic phospholipids. Our observations on the fusion and the bilayer to hexagonal phase transition of DOPE/N-succinyl-DOPE mixtures may provide some insights into the molecular mechanism of this process.

Experimental Procedures

Materials. Egg PC, DOPE, N-NBD-PE, and N-Rho-PE were obtained from Avanti Polar Lipids (Birmingham, AL). N-Succinyl-DOPE was pre-

pared by succinylation of DOPE as described previously [13]. All lipids were chromatographically pure as determined by two-dimensional thin-layer chromatography using activated silica gel 60 thin-layer plates (Merck).

Steady-state fluorescence and light-scattering were quantified at 22°C with a Farrand MKII spectrophotofluorometer. Quasi-elastic light scattering was done using a Nicomp Model 270 Laser Particle Sizer with a 5 mW Helium-Neon Laser at an exciting wavelength of 632.8 nm. Note that the Nicomp device is optimized for the size determination of structures less than one micron in diameter; numerical values greater than this must be accepted with caution.

^{31}P-NMR experiments. The ^{31}P-NMR spectra were obtained employing a Bruker WP-200 spectrometer operating at 81.0 MHz. Free induction decays were accumulated for up to 1000 transients using a radiofrequency (r.f.) pulse width of 11 μs, a sweep width of 50 kHz, a 0.85 s interpulse time and gated high power (> 5 W) broad-band proton decoupling. An exponential filter corresponding to 50 Hz line broadening was applied prior to Fourier transformation.

Samples for ^{31}P-NMR studies were prepared from appropriate mixtures of phospholipid (50 μmol total phospholipid) in chloroform. The chloroform was evaporated under a stream of nitrogen and the sample was then stored under vacuum for 2 h. The lipid was hydrated in 1.0 ml of buffer (100 mM NaCl, 10 mM Hepes (pH 7.4), 10% ^{2}H$_2$O) by vortex mixing. Calcium chloride and/or HCl were added to the lipid mixture from concentrated (100 mM) stock solutions. The samples were freeze-thawed three times to ensure ion equilibrium. Spin-lattice relaxation times were determined using standard inversion-recovery techniques.

Freeze-fracture studies. Freeze-fracture studies were done with a Balzers BAF 400 apparatus. Samples were quenched from 20°C in the presence of 25% glycerol (v/v) and replicas were prepared using standard procedures. The samples were examined with a Joel 1200EX electron microscope.

Fluorescence-energy transfer studies. Membrane fusion was monitored by the probe dilution technique using N-NBD-PE as the energy donor and

N-Rho-PE as the energy acceptor in the same vesicles and unlabeled vesicles as described by Struck et al. [14]. N-Succinyl-DOPE/N-NBD-PE/N-Rho-PE (100 : 1 : 0.5) or DOPE/N-succinyl-DOPE/N-NBD-PE/N-Rho-PE (70 : 30 : 1 : 0.5) unilamellar vesicles (5 μg) prepared by the LUVET procedure [15] were mixed with increasing amounts of unlabeled N-succinyl-DOPE or DOPE/N-succinyl-DOPE LUVETs (0 to 100 μg) in 300 μl of pH 7.4 or 4.0 buffer. Calcium was then added (6 μmol) from a 1 M stock solution and rapidly mixed with the vesicle suspension. After a 30-min incubation at 20°C, the reaction was stopped by the addition of 800 μl of pH 7.2 buffer containing 12 μmol of EDTA. The donor fluorescence intensity at 520 nm (λ_{ex} 450 nm) was determined by using a 520 nm bandpass filter to eliminate light-scattering. Following each measurement, the vesicles were disrupted by the addition of Triton X-100 (2% v/v final concentration) which allowed determination of total N-NBD-PE fluorescence. The energy transfer efficiency was then calculated from the relationship, $E = 1 - (F/F_o)$, where F and F_o are the relative fluorescence intensities in the absence and presence of detergent, respectively. Values obtained in the presence of detergent were corrected for sample dilution and the effect of Triton X-100 on the quantum yield of N-NBD-PE. From the observed transfer efficiencies, the corresponding surface densities of N-Rho-PE was estimated from appropriately generated standard curves [14] at both pH 7.4 and 4.0.

Results

Phase preferences of N-succinyl-DOPE

The polymorphic phase preferences of N-succinyl-DOPE or mixed N-succinyl-DOPE/DOPE systems were investigated by ^{31}P-NMR and freeze-fracture electron microscopy (for review see Ref. 16). Briefly, broad (approx. 40 ppm wide) asymmetric ^{31}P-NMR spectra with a high-field peak and low-field shoulder are observed for large bilayer phospholipid systems, whereas narrower ^{31}P-NMR with reversed asymmetry are observed for hexagonal (H$_{II}$) phase structures. On the other hand, structures which exhibit isotropic motional averaging such as sonicated vesicles, inverted micelles [17], or lipids which adopt the cubic phase [18] give rise to narrow symmetric spectra.

In order to understand the behavior of N-succinyl-DOPE in DOPE/N-succinyl-DOPE systems, it was necessary to characterize the structural preferences of N-succinyl-DOPE alone at pH 7.4 and pH 4.0 in the presence and absence of Ca^{2+}. Fig. 1 shows the ^{31}P-NMR spectra of pure N-succinyl-DOPE liposomes at pH 7.4 and pH 4.0 in the presence and absence of Ca^{2+}. At both pH 7.4 and pH 4.0 (Figs. 1a and 1b, respectively), a broad asymmetric lineshape is observed for which no clearly defined low-field shoulder is observable. This lineshape is similar to the bilayer lineshapes predicted for bilayer phospholipid systems with diameters in the range of 200 to 300 nm [19]. As shown in Fig. 2a, this interpretation is consistent with the freeze-fracture characteristics of N-succinyl-DOPE liposomes, which reveals a major proportion of liposomes with sizes in the range of 100 to 500 nm.

Addition of up to 5-fold molar excess of Ca^{2+} did not alter the lineshape of the N-succinyl-DOPE liposomes at either pH 7.4 or 4.0 (Figs. 1c and 1d) or the freeze-fracture characteristics (Fig. 2b). It has been noted elsewhere that Ca^{2+} also has little effect on unsaturated phosphatidylglycerol dispersions [20] and on phosphatidylinositol dispersions
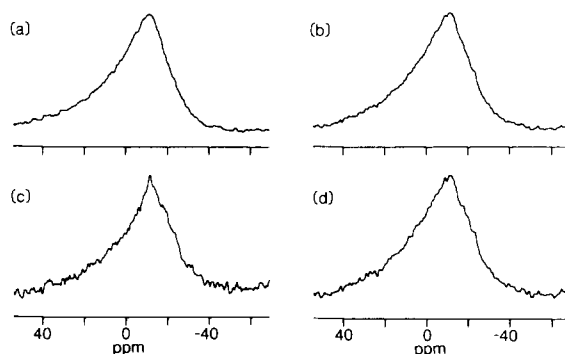

Fig. 1. 81.0 MHz ^{31}P-NMR spectra of N-succinyl-DOPE at 30°C in the presence of excess buffer at (a) pH 7.4; (b) pH 4.0; (c) pH 7.4 in the presence of 5-fold molar excess of calcium; (d) pH 4.0 in the presence of 5-fold molar excess of calcium. Spectra were obtained with 50 μmol phospholipid samples, a 20 kHz sweep width, a 0.8 s interpulse time and gated proton decoupling. Free induction delays were accumulated for up to 1000 transients using a radiofrequency pulse width of 11 μs and a 50 Hz line broadening was applied prior to Fourier transformation.
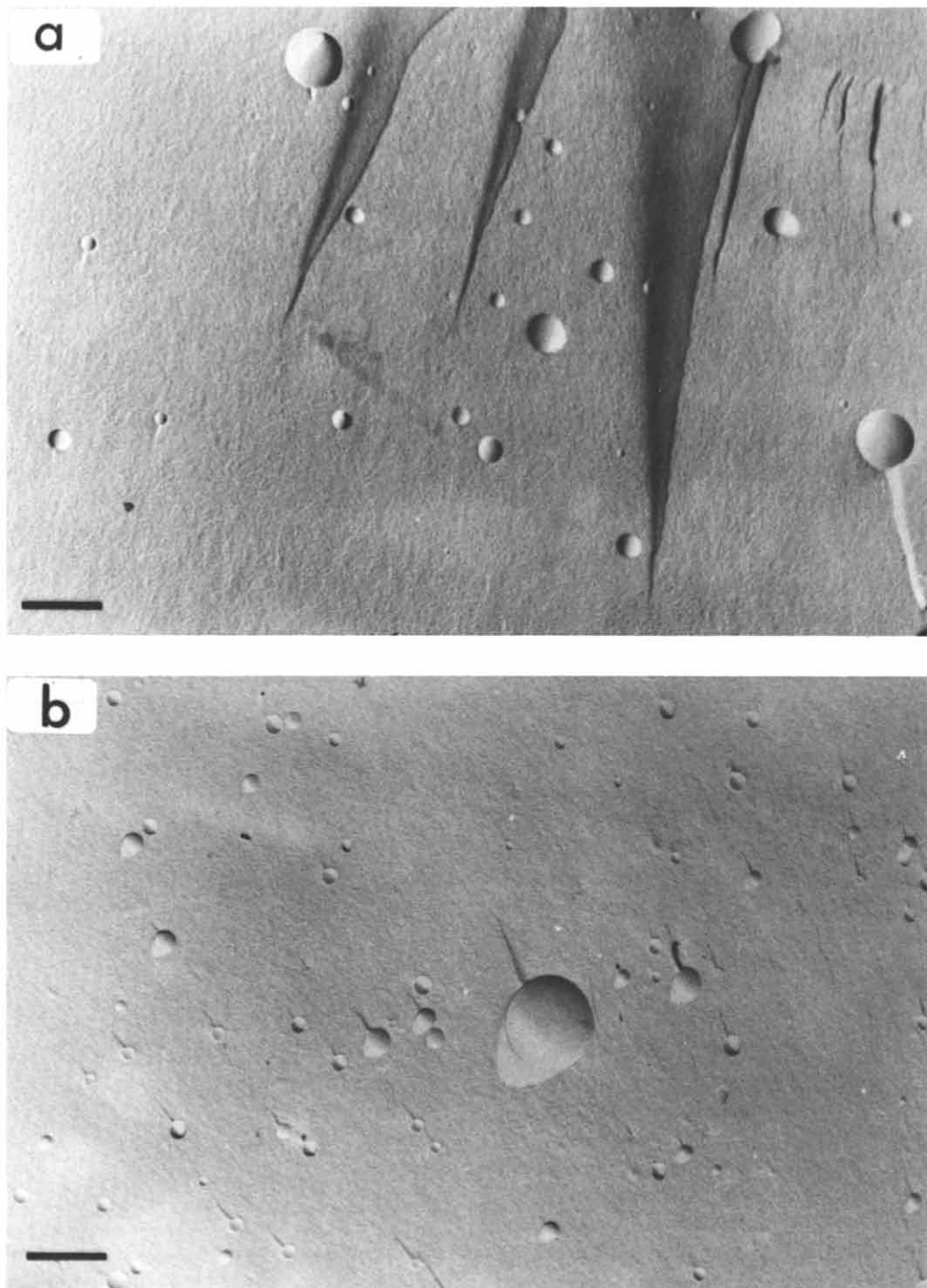
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Fig. 2. Freeze-fracture micrographs of N-succinyl-DOPE MLV. (a) MLV prepared in 150 mM NaCl, 20 mM Hepes, 1 mM EDTA at pH 7.4. (b) MLV in 150 mM NaCl, 20 mM acetate buffer, 1 mM EDTA at pH 4.0 in the presence of 20 mM Ca^{2+}. Bar = 500 nm.

[21]. In other acidic phospholipid systems, Ca^{2+} can induce hexagonal (H_{II}) phase structures in cardiolipin [10] and (unsaturated) phosphatidic acid [8,22] and crystalline 'cochleate' cylinders in phosphatidylserine (PS) dispersions [5]. The inability of Ca^{2+} to induce structural changes in N-succinyl-DOPE may be due, in part, to unsaturation of the acyl chains as it has been noted that increased unsaturation inhibits the ability of Ca^{2+} to induce lateral phase separation in PE-PS mixtures [23].

Alternatively, the inability of divalent cations to trigger H_{II} phase formation could be due to the large effective size of the N-succinylethanolamine head-group.

In order to determine if the addition of Ca^{2+} led to fusion of the pure N-succinyl-DOPE vesicles, freeze-fracture and resonance energy transfer experiments were performed on N-succinyl-DOPE liposomes containing N-NBD-PE and N-Rho-PE. Fusion experiments on unilamellar N-succinyl-DOPE vesicles containing the fluorescent lipid analogues were carried out by mixing the labeled N-succinyl-DOPE vesicles with increasing amounts of unlabeled N-succinyl-DOPE vesicles in the absence and presence of 20 mM Ca^{2+} at pH 7.4 and 4.0. After 30 min, the extent of fusion was estimated by measuring the increase in fluorescent yield of N-NBD-PE, which occurs as a result of reduced energy transfer efficiency caused by an effective decrease in the density of the energy acceptors upon fusion with unlabeled vesicles [14]. The results presented in Fig. 3 show that at an unlabeled to labeled vesicle ratio of 20, the resonance energy transfer efficiency decreased from 66% to 59% at pH 7.2 and to 56% at pH 4.0 in the presence of 20 mM Ca^{2+}. Fusion could not be detected in the absence of calcium (results not shown). Based on a standard curve of N-NBD-PE fluorescence versus the density of the energy acceptor, we estimate that the surface density of N-Rho-PE decreased from 0.0046 (the initial density) to a minimum of 0.0041 and 0.0037 at pH 7.4 and pH 4.0, respectively. This corresponds to fusion of 1 fluorescent vesicle with 0.16 unlabeled vesicles at pH 7.4 and with 0.25 unlabeled vesicles at pH 4.0. This low level of fusion supports the freeze-fracture features of pure N-succinyl-DOPE MLV in the presence of 20 mM Ca^{2+} at pH 4.0
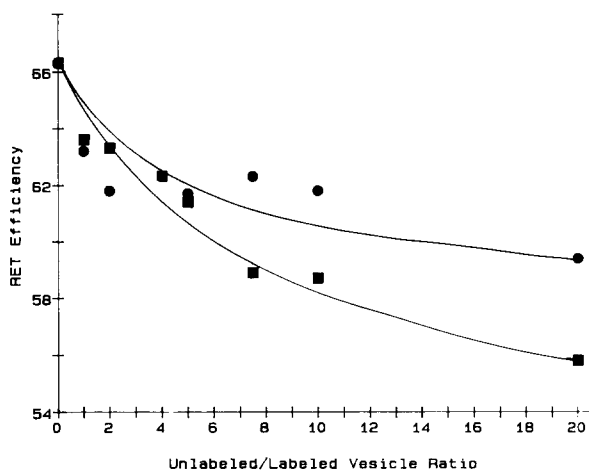

Fig. 3. Effect of pH on fusion of pure N-succinyl-DOPE vesicles in the presence of Ca^{2+}. Fluorescence resonance-energy transfer was monitored by incubating N-succinyl-DOPE vesicles containing 1 mol% N-NBD-PE and 0.5 mol% N-Rho-PE with increasing concentrations of unlabeled vesicles at pH 7.4 and pH 4.0 in the presence of 20 mM Ca^{2+}. The resonance energy transfer efficiency of energy transfer fusion at pH 7.4 (●) and at pH 4.0 (■) was measured as described in Methods.

shown in Fig. 2b, which is very similar to those in the absence of Ca^{2+} (Fig. 2a). Thus, pure N-succinyl-DOPE vesicles do not appear to be particularly fusogenic.

Bilayer stabilizing properties of N-succinyl-DOPE

Previous studies have shown that phospholipids which adopt a bilayer phase (in isolation) can stabilize a bilayer organization in the presence of phosphatidylethanolamine (PE) which would prefer to adopt the H_{II} phase in isolation [24,25]. As shown in Fig. 4 N-succinyl-DOPE can also stabilize the hexagonal H_{II} phase preferring DOPE into a bilayer. The presence of more than 10 mol% N-succinyl-DOPE results in a decrease of the hexagonal component as indicated by the absence of a resonance at 7 ppm which corresponds to the low-field peak from H_{II} phase phospholipids and appearance of a broad lineshape with an isotropic component. Although the cause of the 'isotropic' component cannot be ascertained unambiguously by ^{31}P-NMR, it could be due to lipidic particle structures [17,26], cubic phases [18] or small bilayer vesicles [16].

In the 30 mol% N-succinyl-DOPE, a characteristic bilayer ^{31}P-NMR lineshape which exhibits a
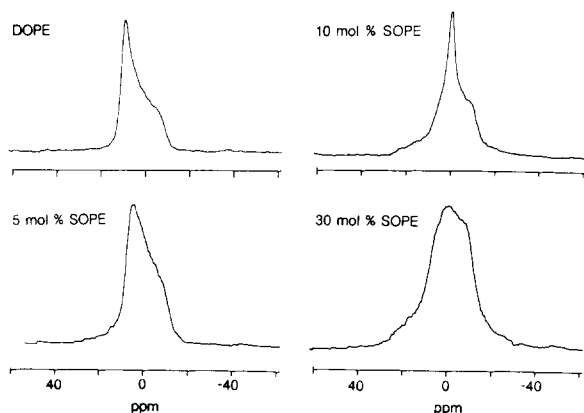
Fig. 4. 81.0 MHz ^{31}P-NMR spectra obtained at 30°C from aqueous dispersions of DOPE/N-succinyl-DOPE mixtures. SOPE, N-succinyl-DOPE.

broad asymmetric resonance together with a narrow isotropic component is observed (Fig. 4). Attempts to identify the source of the narrow component by freeze-fracture analysis revealed small bilayer structures but no lipidic particles as seen in the freeze-fracture micrograph in Fig. 9a.

Effect of calcium on bilayer stabilization

It has been previously shown that Ca^{2+} can trigger bilayer to H$_{II}$ phase transitions in mixtures of PE with acidic phospholipids, such as PS [6,27], cardiolipin [28] and phosphatidylglycerol [20]. As shown in Fig. 5 equimolar Ca^{2+} (with respect to N-succinyl-DOPE) can also trigger H$_{II}$ formation of N-succinyl-DOPE/DOPE dispersions containing from 5 to 30 mol% N-succinyl-DOPE as can be seen by the appearance of a spectrum with reversed asymmetry which is a factor of two narrower than the bilayer lineshape. This is consistent with the H$_{II}$ phase structure.

To investigate the possible mechanism of the lamellar to H$_{II}$ phase transition of these lipids, dispersions were titrated with Ca^{2+} in the presence of the calcium ionophore A23187 (which does not affect the phase behavior) and freeze-thawed to ensure equilibration of the cation. Increasing the Ca^{2+} concentration in 30 mol% N-succinyl-DOPE dispersions resulted in ^{31}P-NMR spectra which reflect a concomitant transition from a bilayer to a hexagonal (H$_{II}$) organization (Fig.
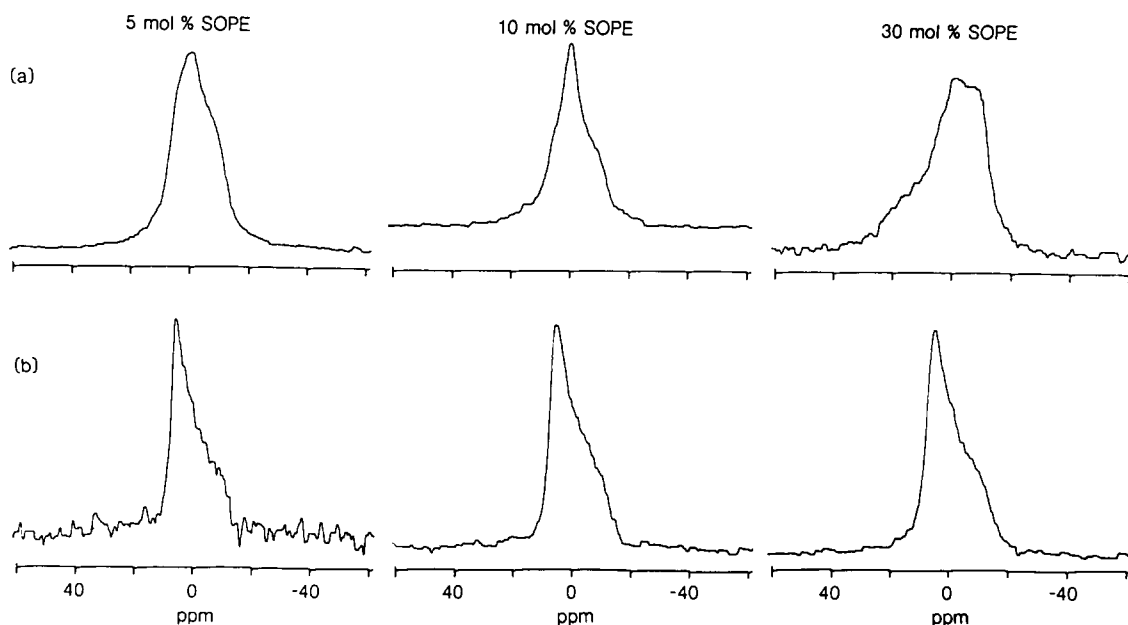

Fig. 5. Influence of calcium on DOPE/N-succinyl-DOPE mixtures. 81.0 MHz ^{31}P-NMR spectra obtained at 30°C from aqueous dispersions of DOPE containing 5, 10, and 30 mol% N-succinyl-DOPE in the absence (a) and presence (b) of Ca^{2+}. Calcium (equimolar with respect to N-succinyl-DOPE) was added and the samples were freeze-thawed three times to equilibrate the ions across the membranes.
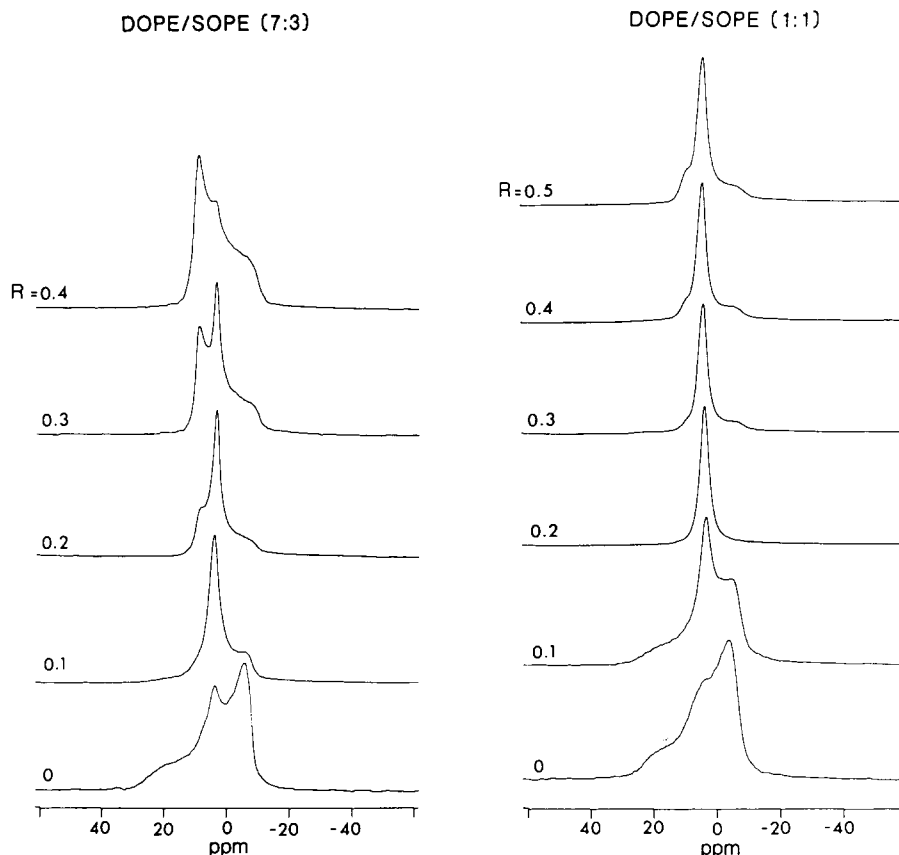
DOPE/SOPE (7:3)

DOPE/SOPE (1:1)

Fig. 6. Calcium titration on DOPE/N-succinyl-DOPE mixtures containing 30 and 50 mol% N-succinyl-DOPE. 81.0 MHz ^{31}P-NMR spectra at 30°C were obtained from aqueous dispersions of liposomes. The ratio, R, refers to the molar ratio of calcium to N-succinyl-DOPE where the divalent cation was added to the hydrated lipid systems as aliquots from a 100 mM stock solution. The calcium ionophore, A23187, was added at 0.1 mol% to aid calcium ion equilibration and the samples were freeze-thawed two times.

6). A narrow spectral feature at 0 ppm (indicative of isotropic motional averaging) appears at Ca^{2+} concentrations (Ca^{2+}/N-succinyl-DOPE = 0.1) below that required to induce predominant H_{II} structures (Ca^{2+}/N-succinyl-DOPE = 0.4). Increasing the amounts of Ca^{2+} in the 50 mol% N-succinyl-DOPE dispersions also induced the appearance of the isotropic ^{31}P-NMR peak, although at high Ca^{2+}/N-succinyl-DOPE ratio ($R = 0.5$) the spectrum remained mainly isotropic with only a small hexagonal H_{II} phase component (Fig. 6).

Effect of pH on N-succinyl-DOPE/DOPE membrane systems

Fig. 7 shows that protonation of the carboxyl moiety of N-succinyl-DOPE at pH 4.0 decreases its ability to stabilize the bilayer resulting in the

appearance of an H_{II} component in the ^{31}P-NMR spectra at both 5 and 10 mol% N-succinyldioleoyl PE. In the case of 30 mol% N-succinyldioleoyl PE, the ^{31}P-NMR lineshape corresponds to a narrow isotropic peak at 0 ppm. The addition of excess Ca^{2+} to 5, 10 and 30 mol% N-succinyldioleoyl PE did, however, trigger H_{II} phase transitions at pH 4.0 as determined by ^{31}P-NMR (results not shown) with spectra similar to those observed at pH 7.4 in the presence of Ca^{2+} (see Fig. 5). To determine if this observation was the result of Ca^{2+}-induced fusion, 30 mol% N-succinyl-DOPE unilamellar vesicles containing N-NBD-PE and N-Rho-PE were mixed with increasing amounts of unlabelled DOPE/N-succinyl-DOPE (7:3) vesicles and fusion was monitored by resonance energy transfer. As shown in Fig. 8 increasing amounts of un-
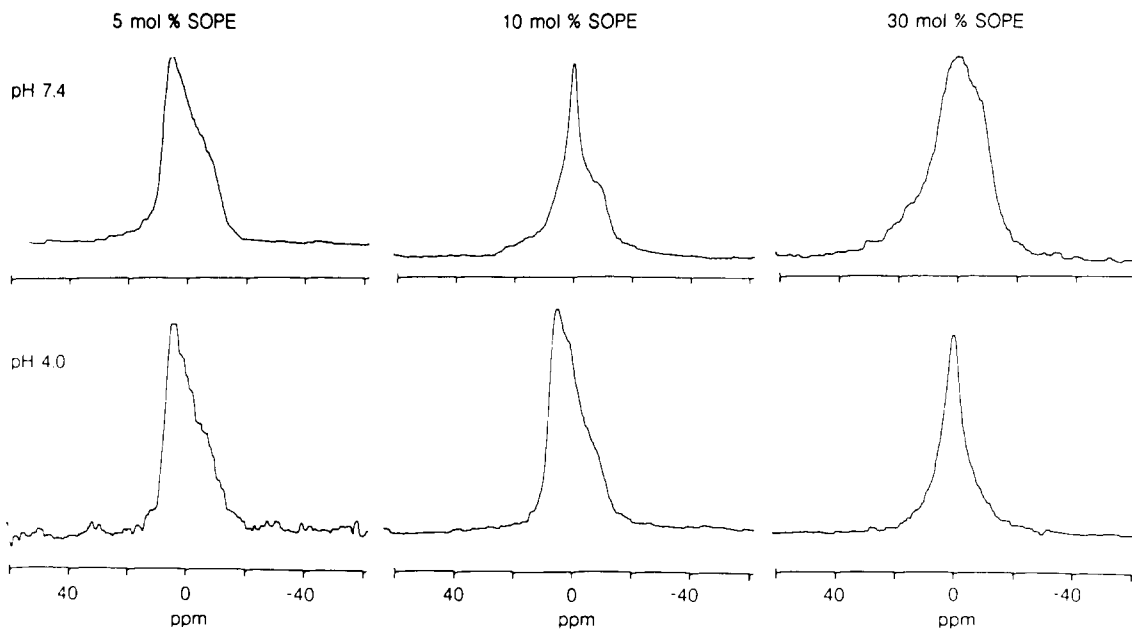
Fig. 7. Effect of pH on bilayer stabilization of DOPE by N-succinyl-DOPE. 81.0 MHz ^{31}P-NMR spectra were obtained from aqueous dispersions of DOPE containing 5, 10, and 30 mol% N-succinyl-DOPE at pH 7.4 and 4.0. The pH was adjusted by addition of aliquots from 100 mM HCl stock solutions to the lipid dispersions followed by three freeze-thaw cycles.

labeled acceptors resulted in decreased efficiency of energy transfer due to a corresponding decrease in probe density (Fig. 8a). Fig. 8b shows the effect of Ca^{2+} concentration on fusion of labeled DOPE/N-succinyl-DOPE vesicles in the presence of 10-fold excess unlabeled DOPE/N-succinyl-DOPE vesicles. The fusion of DOPE/N-succinyl-DOPE vesicles proceeds more readily at pH 4.0

than at pH 7.4. The vesicles fused readily with 1.0 mM Ca^{2+} at pH 4.0 whereas more than 10 mM Ca^{2+} was required at pH 7.4 to achieve a similar degree of fusion. In the absence of Ca^{2+}, energy transfer efficiency decreased from 64% to 34% suggesting Ca^{2+}-independent pH-induced fusion at pH 4.0. Such a fusion event was also seen in freeze-fracture analysis as shown in Fig. 9b.
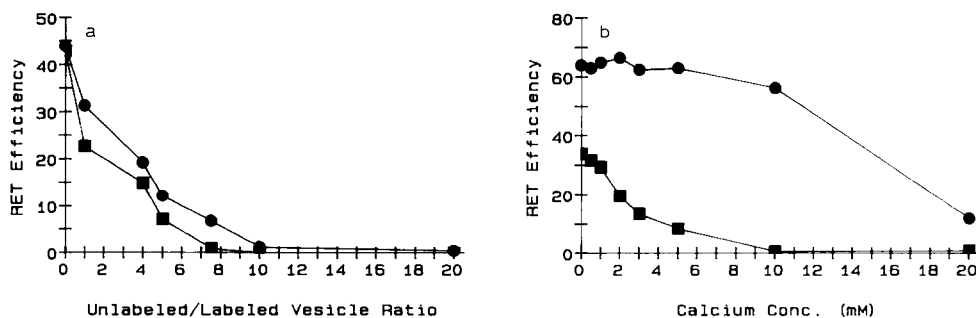

Fig. 8. Effect of calcium and pH on fusion of DOPE/N-succinyl-DOPE vesicles. (a) resonance energy transfer efficiency was monitored by incubating DOPE/N-succinyl-DOPE (7:3, mol ratio) vesicles containing 1 mol% of N-NBD-PE and 0.5 mol% N-Rho-PE in the presence of increasing amounts of unlabeled DOPE/N-succinyl-DOPE vesicles in pH 7.4 (●) and 4.0 (■) buffers containing 20 mM Ca. (b) Calcium titration of fusion was done at an unlabeled/labeled vesicle ratio of 10 at pH 7.4 (●) and pH 4.0 (■). The reaction was stopped after a 30 min incubation by the addition of 2-fold molar excess EDTA (adjusted to pH 7.4) and resonance energy transfer efficiency was determined.
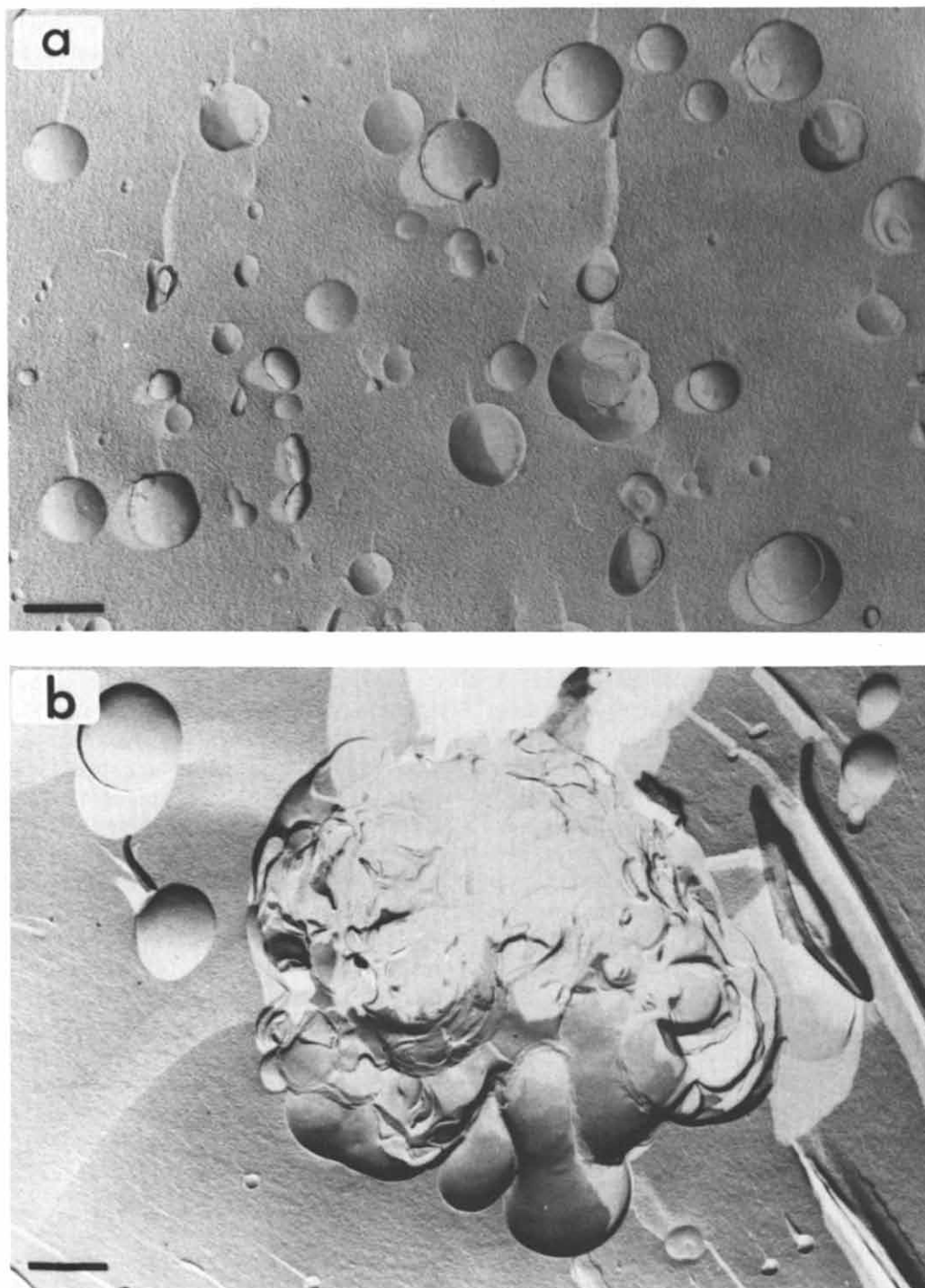
Fig. 9. Freeze-fracture micrographs of DOPE/N-succinyl-DOPE MLV containing 30 mol% N-succinyl-DOPE. (a) MLV prepared in 150 mM NaCl, 20 mM Hepes, 1 mM EDTA at pH 7.4. (b) MLV in 150 mM NaCl, 20 mM acetate buffer, 1 mM EDTA at pH 4.0. Bar = 500 nm.

Discussion

The object of this study was to characterize the structural preferences of N-succinyl-DOPE in pure and mixed model systems. It is shown that pure N-succinyl-DOPE liposomes exhibit polymorphic behavior which is very similar to that observed for other acidic phospholipids. That is, N-succinyl-DOPE prefers a lamellar organization on hydration as indicated by ^{31}P-NMR and freeze-fracture techniques. With the addition of excess calcium, the ^{31}P-NMR spectra and freeze-fracture analysis indicate that the bilayer structure is maintained as also has been observed for phosphatidylinositol [21]. Furthermore, protonation of the succinyl group of N-succinyl-DOPE did not alter the polymorphic behavior of the lipid in the presence or absence of calcium.

In mixed dioleoyl PE systems, N-succinyl-DOPE is an effective agent for stabilizing the DOPE into a bilayer conformation at more than 10 mol% N-succinyl-DOPE. The subsequent addition of calcium to these systems destabilized the bilayer and induced the hexagonal H_{II} phase in multilamellar systems. In unilamellar systems containing 30 mol% N-succinyl-DOPE, Ca^{2+} stimulated membrane fusion events as detected by fluorescence energy transfer. This behavior is consistent with that observed for other acidic phospholipid-PE systems on addition of Ca^{2+} [11], but differs from the cholesteryl hemisuccinate-PE systems where the lamellar structure is maintained [29]. It is unlikely that formation of the hexagonal H_{II} phase in the DOPE/N-succinyl-DOPE system is a result of calcium-induced lateral phase separation of N-succinyl-DOPE thereby allowing the DOPE molecules to adopt the hexagonal H_{II} phase for the following reason. The spin-lattice relaxation time for N-succinyl-DOPE is approximately 0.2 s, both in the presence and absence of calcium at both pH 7.4 and 4.0. Thus, as previously discussed [6], we would expect to observe greater than 98% of the signal of N-succinyl-DOPE in the NMR spectrum under our experimental conditions. If a calcium-induced lateral phase separation of the DOPE and N-succinyl-DOPE had occurred, thereby leaving the DOPE free to adopt the hexagonal H_{II} phase, then a H_{II} phase DOPE NMR component should be superimposed on a bilayer phase N-succinyl-DOPE component. Such a result was never observed, indicating that phase separation did not occur.

Previous studies have noted the close correlation between the ability of lipid systems to adopt the hexagonal H_{II} phase and membrane fusion processes (for review, see Ref. 30). In the case of Ca^{2+}-induced effects for example, the ability of Ca^{2+} to induce H_{II} phase in multilamellar systems is correlated to an ability of Ca^{2+} to fuse unilamellar systems with the same lipid composition. Two mechanisms for such fusion processes have been proposed. The first involves Ca^{2+} segregation of acidic lipids to provide a nucleation point at the fusion interface [5] whereas the second proposes formation of a transient non-bilayer intermediate (such as an inverted micelle) [24,30,31]. The lack of Ca^{2+}-induced separation of the N-succinyl-DOPE component would suggest that fusion of the DOPE/N-succinyl-DOPE systems are more consistent with the latter possibility.

In summary, N-succinyl-DOPE's polymorphic phase properties are similar to other acidic phospholipids. That is, (i) it assumes a bilayer organization in isolation, (ii) it can stabilize non-bilayer phospholipids into a bilayer organization, and (iii) calcium can trigger bilayer to hexagonal H_{II} phase transition in mixed lipid systems containing DOPE. This transition is related to Ca^{2+}-stimulated membrane fusion events observed in unilamellar DOPE/N-succinyl-DOPE vesicle systems. As the pH is lowered towards the pK_a of the succinyl carboxyl, such transitions proceed more readily, suggesting the utility of N-succinyl-DOPE-containing systems in drug delivery application as fusion is likely to be enhanced in acidic environments such as the endosome. This mode of drug delivery would thus facilitate more efficient cytoplasmic delivery of the encapsulated drugs.

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