Acid- and Calcium-Induced Structural Changes in Phosphatidylethanolamine Membranes Stabilized by Cholesteryl Hemisuccinate[†]

Ming-Zong Lai,[‡] William J. Vail,[§] and Francis C. Szoka*[,‡]

Department of Pharmacy and Pharmaceutical Chemistry, University of California, San Francisco, California 94143, and Department of Biology, Frostburg State College, Frostburg, Maryland 21532

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ABSTRACT: The membrane stabilization effect of cholesteryl hemisuccinate (CHEMS) and the sensitivity of the CHEMS–phosphatidylethanolamine membranes to protons and calcium ions were studied by differential scanning calorimetry, freeze–fracture electron microscopy, and ^{31}P NMR. (1) At neutral pH, the addition of 8 mol % CHEMS to transesterified egg phosphatidylethanolamine (TPE) raised the lamellar–hexagonal transition temperature of TPE by 11 °C. Stable bilayer vesicles were formed when the incorporated CHEMS exceeded 20 mol %. (2) At a pH below 5.5, the protonation of CHEMS enhanced the formation of the hexagonal phase (H_{II}) of TPE. At 25 mol % CHEMS the bilayer–hexagonal transition temperature was lowered by 30 °C at pH 4.5. (3) The endothermic acid-induced hexagonal transition of TPE–CHEMS was suppressed at 35 mol % CHEMS. However, ^{31}P NMR and electron microscopy indicated that a lamellar–hexagonal transition still occurred at this composition. (4) The main transition of TPE was not affected by the protonation of the incorporated CHEMS, indicating that no macroscopic phase separation occurred in TPE–CHEMS mixtures at low pH. (5) In contrast to the H_{II}-promoting effect of H^+, the neutralization of the negative charge on TPE–CHEMS by Ca^{2+} resulted in aggregates that remained in the lamellar structure even at the hexagonal transition temperature of TPE. It is suggested that calcium might form a complex between CHEMS in apposed bilayers. These results are related to the possible biological function of acidic cholesterol esters in biomembranes.

Negatively charged phospholipids such as phosphatidylserine and phosphatidylglycerol have been implicated in important biological functions of biomembranes because the structure and properties of these anionic phospholipids are charge dependent. The order–fluid transition of acidic phospholipids can be regulated by changes in pH or divalent ion concentration (Trauble & Eibl, 1974; Trauble, 1976). Anionic phospholipids neutralized with protons or calcium are highly ordered (Newton et al., 1978; Harlos & Eibl, 1980a; Liao & Prestegard, 1981) and are segregated from other membrane lipids, resulting in the destabilization of the membranes (Jacobson & Papahadjopoulos, 1975; Galla & Sackmann, 1975; Tokutomi et al., 1980). The interaction of protons, as well as divalent or polyvalent cations, with acidic phospholipids also leads to membrane fusion (Papahadjopoulos et al., 1974, 1976, 1977).

While anionic phospholipids have been subjected to extensive study, other acidic lipids have received little attention. Recent work in this and other laboratories have demonstrated interesting properties of cholesteryl phosphate (Colombat et al., 1981) and cholesteryl hemisuccinate (Ellens et al., 1984a; Lai et al., 1985). Despite the lack of the 3β-OH group these cholesterol esters retain the properties and function of cholesterol (Colombat et al., 1981; Lai et al., 1985). Among other anionic cholesterol esters, cholesteryl sulfate is probably an important example; cholesteryl sulfate has been found in brain, adrenal, aorta, liver, kidney, and plasma (Moser et al., 1966) and is located in the intestinal brush border membrane in abundant amounts (Pascher & Sundell, 1977). In human erythrocyte membranes, cholesteryl sulfate has been shown to protect against hypotonic hemolysis (Bleau et al., 1974). On the basis of the specific uptake of cholesteryl sulfate by spermatozoa, Langlais et al. (1981) hypothesized a role for cholesteryl sulfate in membrane stabilization and enzyme inhibition during sperm storage within the epididymis and with the cleavage of sulfate moiety leading to sperm capacitation and fertilization. The pathological state in patients with recessive X-linked ichthyosis has been suggested to be related to the increased content of cholesteryl sulfate in the stratum corneum, resulting from a cholesterol sulfatase deficiency in the epidermis (Williams & Elias, 1981). Thus, negatively charged cholesterol esters are probably playing important roles, similar to those of acidic phospholipids, in the structure and functions of biomembranes.

We have used cholesteryl hemisuccinate (CHEMS)[1] as a model compound to study the interaction between phospholipids and charged cholesterol esters (Lai et al., 1984). In the studies described here, we have also used CHEMS to demonstrate the membrane stabilization effect of anionic cholesterol esters and to study pH- and Ca^{2+}-induced structural changes in membranes containing CHEMS. We have found that protons and calcium have different effects on the phase

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[‡] University of California.

[§] Frostburg State College.

[1] Abbreviations: CHEMS, cholesteryl hemisuccinate; DMPE, dimyristoylphosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DSC, differential scanning calorimetry; EPC, egg phosphatidylcholine; EM, electron microscopy; H_{II} phase, hexagonal phase; L–H transition, lamellar to hexagonal phase transition; PE, phosphatidylethanolamine; PA, phosphatidic acid; PG, phosphatidylglyerol; PS, phosphatidylserine; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; TPE, phosphatidylethanolamine prepared from egg phosphatidylcholine by transesterification; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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behavior of CHEMS–PE liposomes. The response of PE–CHEMS membranes to protons and calcium ions may be related to the biological functions of acidic cholesterol esters.

MATERIALS AND METHODS

Lipids and Chemicals. Egg phosphatidylcholine (EPC), transesterified egg phosphatidylethanolmine (TPE), and dioleoylphosphatidylethanolmine (DOPE) were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesteryl hemisuccinate was purchased from Sigma. All lipids produced a single spot in silica gel thin-layer chromatography with chloroform/methanol/acetic acid/water (100/50/14/16) and chloroform/methanol/ammonia/water (115/45/2/6) and were stored under nitrogen at −50 °C. D_2O was obtained from Aldrich. All other chemicals were of reagent grade or better.

Aqueous Dispersions of Lipids. Lipids were first deposited onto a round-bottom flask by removing the organic solvent through rotatory evaporation. Lipids were then hydrated in Tris-HCl–NaCl buffer (50 mM Tris-HCl and 100 mM NaCl, pH 7.4) under positive nitrogen pressure. Brief sonication (2–5 min) was applied to lipids that did not easily hydrate. The pH was adjusted by dialyzing against buffer (50 mM acetate and 110 mM NaCl, pH 3.5–5.5), or by direct mixing with a large volume (>10×) of concentrated acidic buffer (150 mM acetate and 25 mM NaCl). The final pH of the dispersions was checked with two sets of Hydrion Microfine pH test paper (Micro Essential Laboratory, NY) with overlapping pH ranges. The pH for all samples was measured at 25 °C. The Ca^{2+} concentration was altered by dialyzing against Tris-HCl–NaCl buffer containing calcium. To minimize the oxidation of phospholipids, samples were freshly prepared for each study, and dialysis was performed with argon bubbling through the buffer.

Differential Scanning Calorimetry. Samples for DSC were concentrated by centrifugation in an Eppendorf centrifuge (12800*g*, 1 min), and the pellet was dispersed in 50 μL of the same buffer used for sample preparation; 17 μL of the final dispersion was sealed in an aluminum sample pan. The lipids precipitated by acid and calcium ions were difficult to redisperse in buffer and were applied directly onto the sample pan followed by the appropriate amount of acidic or Ca^{2+}-containing buffer. DSC measurements were made with a Perkin-Elmer DSC-2 calorimeter operating at a sensitivity of 1 mcal/s and a scanning rate of 5 °C/min. Continuous heating (two) and cooling (one) runs were performed on each sample. At least two samples were studied for each composition of lipid mixtures. Unless specified, no cryoprotectants were added to the sample, and each scan started at 265 K. Because the phase transition of phospholipids of natural origin is broad, the maximum of the excess heat curve was taken as the transition temperature.

Phosphorus-31 Nuclear Magnetic Resonance. Lipid samples for ^{31}P NMR were deposited on the vessel by removing the chloroform in a rotatory evaporator at reduced pressure followed by an additional hour under high vacuum and then hydrated in Tris-HCl–NaCl buffer containing 10% D_2O at a final concentration of 40–50 μmol of phospholipid/mL. For samples where additional dialysis was required for the adjustment of the pH or Ca^{2+} concentration, D_2O was added after dialysis. ^{31}P NMR spectra were recorded on a Varian XL-100 spectrometer operating in the Fourier transform mode at 40.5 MHz. Samples were placed in 12-mm tubes and equilibrated at the temperature to be studied for at least 10 min before signal acquisition. Accumulation free induction decays were obtained from 3000–6000 transients with a 45° pulse angle, 0.5-s interpulse time, and a delay time of 200 μs. A sweep width of 10 kHz was employed, and 4K data points were collected. Exponential multiplication corresponding to a 100-Hz line broadening was applied during signal enhancement. H_3PO_4 (85%) in D_2O (10%) was used as an external standard.

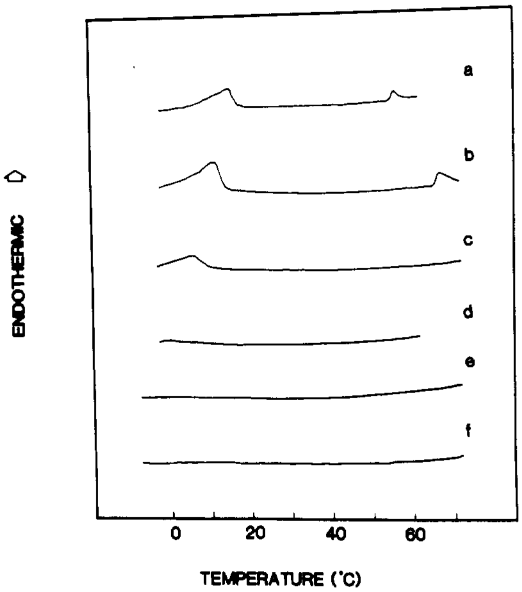

FIGURE 1: Differential scanning calorimetry thermograms of the TPE–CHEMS mixtures at pH 7.4 with (a) 0, (b) 8, (c) 16, (d) 25, (e) 30, and (f) 35 mol % CHEMS.

Freeze–Fracture Electron Microscopy. Samples for freeze–fracture electron microscopy contained 30% v/v glycerol as a cryoprotectant. The lipids precipitated by acid or calcium ion were first spun down in an Eppendorf centrifuge, and pellets were mixed with equal volume of 60% glycerol in the given buffer. Final samples of less than 300 μL were incubated in a water bath at the designated temperature for at least 10 min and were then quickly placed on gold cups which were on a hot plate at a temperature 5 °C higher than that of the water bath. The cups were immediately frozen in Freon 22 and stored in liquid nitrogen. Samples were fractured in a Balzer BA 360M freeze etch unit at −115 °C under a vacuum below 10^{-6} torr and replicated with carbon–platinum and then with carbon. Replicas were floated on water, washed for 60 min in commercial Chlorax and twice in water, and mounted on bare 75 × 300 copper grids. The residual lipids were removed by placing over boiling chloroform and allowing vapors to condense on the grids (Vail & Stollery, 1978). Specimens were examined in a Seimens Elmiskop 1A electron microscope.

RESULTS

Aqueous Dispersions of TPE. The dispersion of transesterified egg phosphatidylethanolamine (TPE) in Tris-HCl–NaCl buffer (pH 7.4) precipitated rapidly to form large aggregates. Similar observations have been reported on egg phosphatidylethanolamine (Papahadjopoulos & Miller, 1967; Littman, 1973), dimyristoylphosphatidylethanolamine, and dipalmitoylphosphatidylethanolamine (Kolber & Haynes, 1979). In the DSC scan (Figure 1a) TPE displayed an order–fluid transition at 15 °C and a second transition at 56 °C which has been correlated with bilayer–hexagonal transition (Boggs et al., 1981). The transition temperature of TPE is highly dependent on the source; a difference of 2 °C in the main transition and 6 °C in hexagonal transition has been observed with different batches of TPE. Since all TPE were shown to be free of oxidized products on thin-layer chroma-

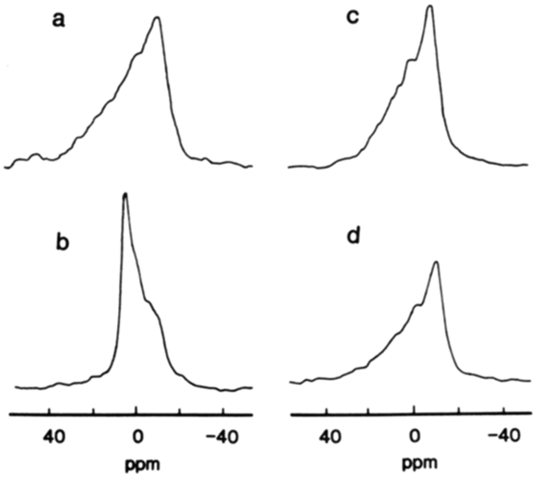

FIGURE 2: Proton-decoupled ^{31}P NMR spectra at 40.5 MHz of the aqueous dispersions of TPE and TPE and TPE–CHEMS at pH 7.4. (a) TPE at 50 °C, (b) TPE at 61 °C, (c) TPE–CHEMS (75/25) at 65 °C, and (d) TPE–CHEMS (58/42) at 65 °C.

tography, the difference in the transition are most likely due to slight variation in the acyl chain composition of natural phospholipids. This might explain the discrepancy in the transition temperature reported from different laboratories (Boggs et al., 1981; Mantsch et al., 1981). The ^{31}P NMR spectrum characteristic of a lamellar phase was converted into a spectrum of a hexagonal phase in the temperature range corresponding to the second transition peak on the thermoscan (Figure 2a,b). However, attempts to capture the hexagonal phase of TPE on freeze–fracture EM failed even when the temperature was raised to 80 °C. A similar observation has been reported before (Hui et al., 1981) and could be due to the difficulties in preserving the hexagonal structure at high temperature (Verkleij & de Gier, 1981). The freeze–fracture EM of TPE at 65 °C showed fused lamellar structures with some in a concentric form (Figure 3a).

Stabilization of TPE in Vesicular Form by CHEMS at pH 7.4. The addition of CHEMS to TPE altered the main transition of TPE in a similar fashion to that observed previously for DMPE–CHEMS mixtures (Lai et al., 1984). The incorporation of 8 mol % CHEMS decreased the main transition by 4 °C; increasing the molar ratio of CHEMS shifted the order–fluid transition to a lower temperature (Figure 1b,c). The main transition was barely detectable with 25 mol % CHEMS and was completely suppressed as the content of CHEMS was increased to 30 mol % (Figure 1d,e). The opposite trend was seen on the lamellar–hexagonal transition of TPE as the CHEMS mole ratio was increased. At 8 mol % CHEMS, the peak temperature of L–H transition was moved to 67 °C, 11 °C higher than the hexagonal formation temperature of TPE (Figure 1b). At greater ratios of CHEMS, the L–H transition was not detectable even up to 72 °C (Figure 1c–e), which is also confirmed by the NMR spectra (Figure 2c,d). Multilamellar vesicles of TPE can be easily prepared with 20 mol % or more of CHEMS. Freeze–fracture EM showed that the vesicular structure was retained at temperatures above the hexagonal transition temperature of TPE (Figure 3b) with the appearance of a few lipidic particles. Reverse-phase evaporation vesicles (Ellens et al., 1984a) and small unilamellar vesicles were also readily prepared at these molar ratios (electron microscopy not shown).

Effect of pH on PE–CHEMS Vesicles. The CHEMS–TPE membranes were acidified to study the effect of protonation on the carboxylate group of CHEMS. TPE by itself is insensitive to the pH change in this region (Figure 4a; Trauble & Eibl, 1974). Both the order–fluid and lamellar–hexagonal transitions were only slightly affected when the pH was shifted to 4.5 (Figure 4a). Parts a and b of Figure 5 show the ^{31}P NMR spectra of TPE at pH 4.5, confirming that the L–H transition occurred in the same temperature region. The change of pH from 7.4 to 4.5 also had a minimal effect on the main transitions of the TPE–CHEMS mixtures (Figure 4b–e); the shifts of the peak transition temperatures were less

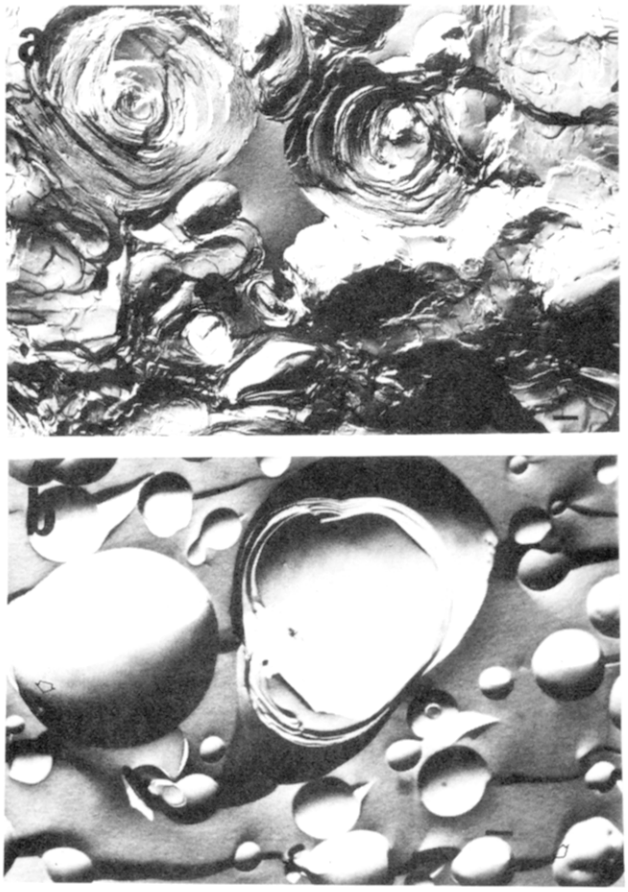

FIGURE 3: Freeze–fracture electron micrograph of the aqueous dispersions of TPE and TPE–CHEMS at pH 7.4. (a) TPE at 65 °C. (b) TPE–CHEMS (75–25) at 65 °C. Bar is 0.2 μm. Arrow in (b) indicates lipidic particles.

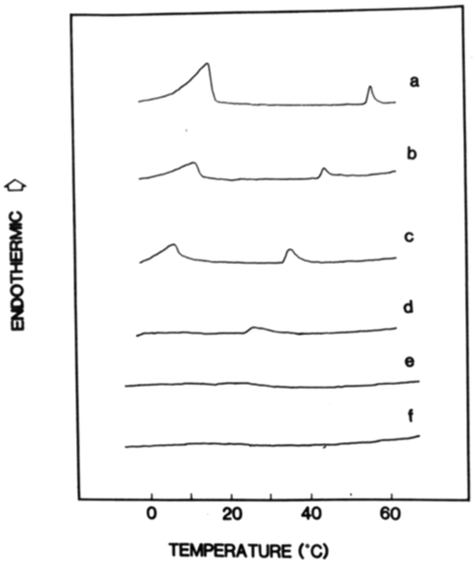

FIGURE 4: Differential scanning calorimetry thermoscan of the TPE–CHEMS mixtures at pH 4.5 with (a) 0, (b) 8, (c) 16, (d) 25, (e) 30, and (f) 35 mol % CHEMS.

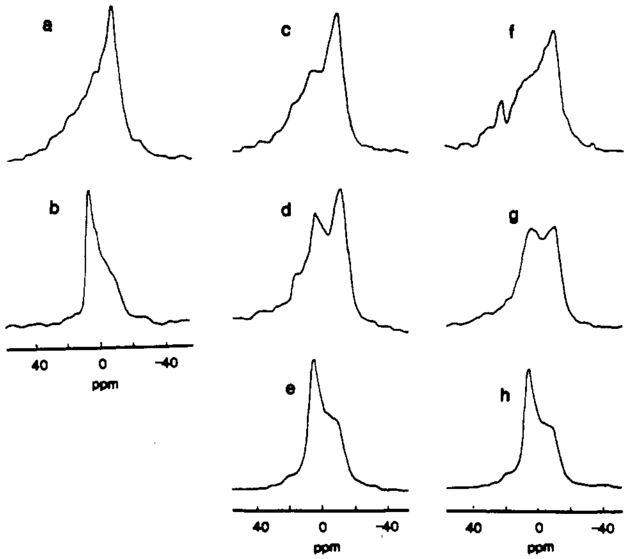

FIGURE 5: Proton-decoupled ^{31}P NMR spectra at 40.5 MHz of the lipids at pH 4.5. (a) TPE at 50 °C, (b) TPE at 60 °C, (c) TPE–CHEMS (75/25) at 18 °C, (d) TPE–CHEMS (75/25) at 24 °C, (e) TPE–CHEMS (75/25) at 30 °C, (f) TPE–CHEMS (65/35) at 5 °C, (g) TPE–CHEMS (65/35) at 10 °C, and (h) TPE–CHEMS (65/35) at 18 °C.

than 0.5 °C. Since proton-induced phase separation of PC/PS membranes occurs slowly with an equilibrium time of 2 h at pH 2.5 (Tokutomi et al., 1980), to determine if the phase separation of TPE–CHEMS (70/30) is also a slow process, we allowed the system to equilibrate for 24 h at low pH. Under this condition, the scan was identical to that before equilibrium. On the other hand, the L–H transitions of TPE–CHEMS mixtures were highly acid sensitive. For TPE–CHEMS of 92/8 composition at pH 4.5, the peak hexagonal transition temperature was shifted to 43 °C (Figure 4b), a 24 °C downshift from the corresponding transition peak at neutral pH and 13 °C lower than that of pure TPE. The shift in the L–H transition was also dependent on the acid concentration; the transition temperature at pH 4.5 is 5 and 1 °C, respectively, lower than that at pH 5.5 and 5.0 (Figure 6a–c), implying that the promotion of the H_{II} phase is related to the protonation of CHEMS. In addition, the more CHEMS in the TPE mixtures, the lower the temperature at which the acid-induced hexagonal transition takes place (Figure 4c,d). For TPE containing 25 mol % CHEMS, the peak hexagonal transition temperature is 26 °C at pH 4.5. This is a temperature 30 °C lower than the L–H transition temperature of TPE. ^{31}P NMR spectra also indicated a hexagonal transition in this temperature region (Figure 5c–e). The presence of the hexagonal phase at 37 °C was confirmed by freeze–fractue EM (Figure 7a), showing stacked lipid cylinders running parallel to each other. The L–H transition temperatures of the mixture in 30% glycerol (for freeze–fracture), however, were always 8–11 °C lower than that of the same mixture in the absence of glycerol. At 22 °C the acidified TPE–CHEMS (75/25) remained in hexagonal and hexagonal-like structures (Figure 7b). Electron microscopy of lamellar structures could be observed only when the TPE–CHEMS mixture was incubated at temperatures below 10 °C. Figure 7c shows the structure of aggregated vesicles of TPE–CHEMS (75/25) at 4 °C and pH 4.5. Such a H_{II}-promoting effect by glycerol can be easily observed in the thermoscan of TPE with 8 mol % CHEMS (Figure 6e). Similar shifts in endothermic H_{II} transition were found with TPE–CHEMS at 82/16 and 75/25 compositions containing 30% glycerol (scan not shown).

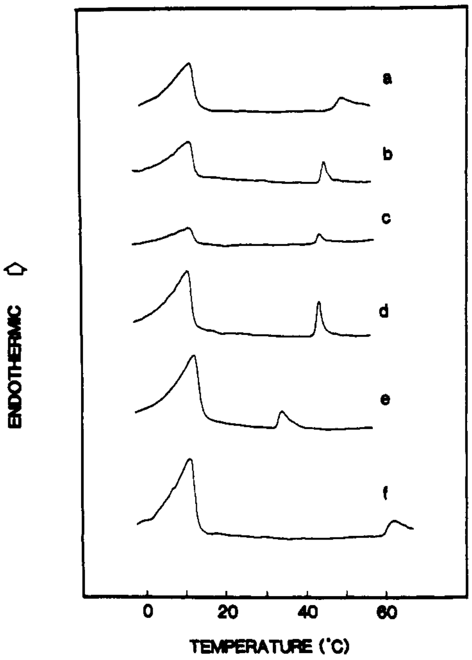

FIGURE 6: Thermoscans of TPE–CHEMS (92/8) at (a) pH 5.5, (b) pH 5.0, (c) pH 4.5, (d) pH 4.0, (e) pH 4.5 with 30% glycerol, and (f) pH 7.4 with 3 mM Ca^{2+}.

At 30 mol % CHEMS the endothermic L–H transition induced by acid was barely detected between 17 and 28 °C (Figure 4e). The acid-induced hexagonal transition was not detected on DSC as the content of CHEMS in TPE mixtures reached 35 mol % (Figure 4f). NMR spectra indicated that the lipid mixture still underwent a lamellar–hexagonal transition at temperatures between 5 and 18 °C (Figure 5f–h). The hexagonal structure was also demonstrated by freeze–fracture EM for TPE containing higher amounts of CHEMS (Figure 7d).

The membrane stabilization effect of CHEMS was also studied with DOPE. DOPE has a hexagonal transition temperature in the range 10–18 °C (van Dijck et al., 1976). At 25 °C, the addition of CHEMS in excess of 20 mol % stabilized DOPE in the vesicle form. Acidification of the vesicles resulted in the reappearance of the hexagonal phase as detected by freeze–fracture EM and ^{31}P NMR (data not shown).

In contrast to PE membranes, PC membranes containing CHEMS were only slightly affected by acidification. Upon exposure to pH 4.5 buffer, liposomes composed of EPC–CHEMS (7/3) slowly aggregated. The aggregates could be redispersed, and freeze–fracture EM demonstrated the dispersion remained in the form of vesicles at 65 °C (Figure 7e).

Effect of Calcium on PE–CHEMS Mixtures. Addition of calcium ions to TPE–CHEMS (7/3) liposomes also aggregated the vesicles and released the contents of the vesicles in a way similar to the low pH effect (Ellens et al., 1984b). Examination of the aggregated lipid with ^{31}P NMR indicated that the mixture was in the lamellar phase at 25 °C (Figure 8a). The ^{31}P NMR spectra characteristic of a lamellar phase predominated even on heating to 65 °C (Figure 8b,c), the temperature at which TPE should be in the hexagonal phase. The hexagonal phase did not appear until 78 °C (Figure 8d). The freeze–fracture EM showed aggregated vesicles, some with a much larger diameter (Figure 9b) than that of the original preparation (Figure 9a). The Ca^{2+}-aggregated structures are in contrast to the H_{II} structures of the TPE–CHEMS mixtures of similar compositions at the same temperature (Figure 7c,e). The thermoscan of the same mixture (scan not shown) is identical with that in the absence of Ca^{2+} (Figure 1e) between

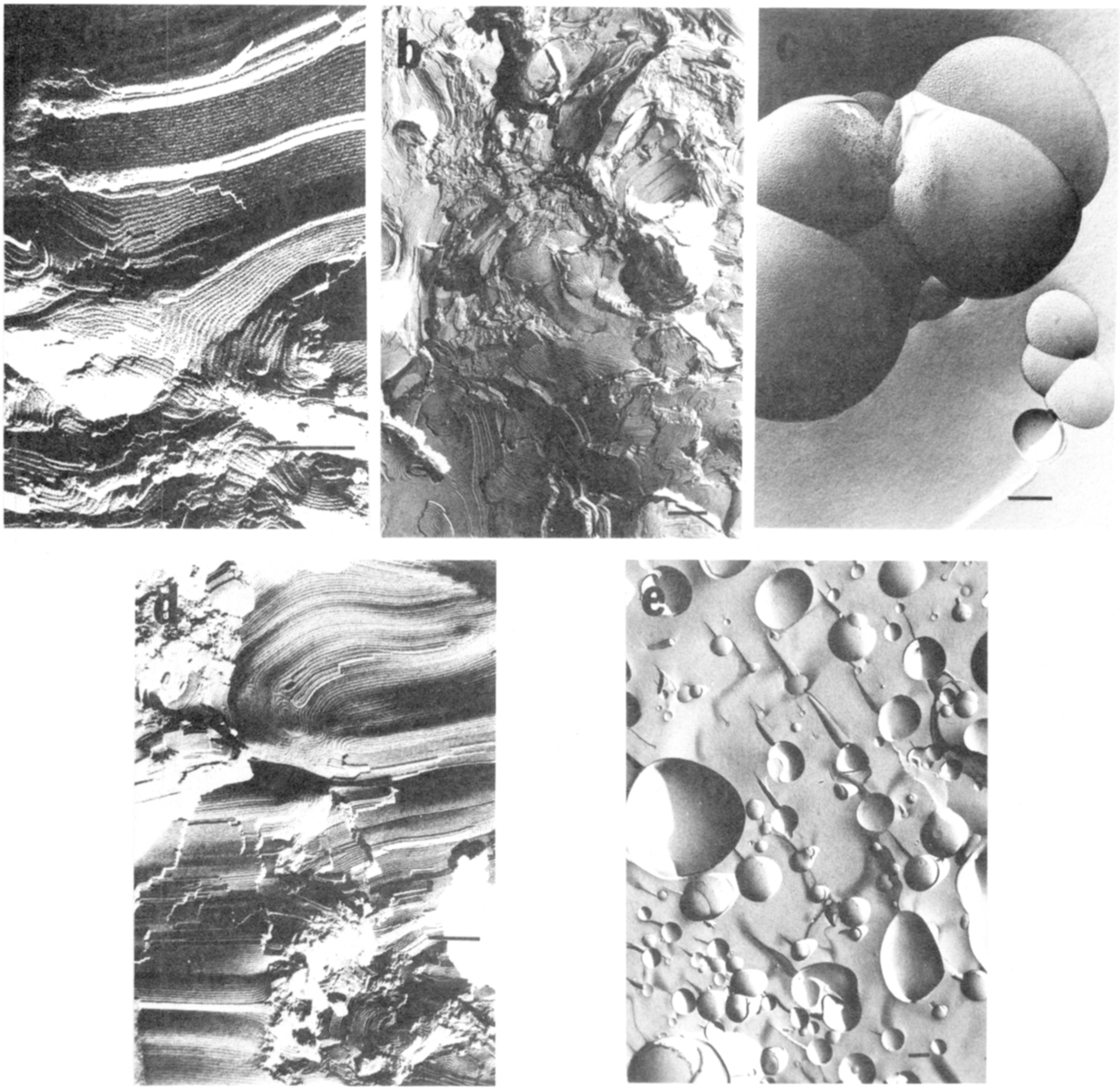

FIGURE 7: Freeze–fracture electron micrograph of the lipids at pH 4.5. (a) TPE–CHEMS (75/25) at 37 °C. (b) TPE–CHEMS (75/25) at 22 °C. (c) TPE–CHEMS (75/25) at 4 °C. (d) TPE–CHEMS (58/42) at 20 °C. (e) EPC–CHEMS (70/30) at 65 °C. Bar is 0.2 μm.

−8 and 72 °C. The main transtion of TPE remained suppressed in the presence of Ca^{2+}, indicating that macroscopic phase separation did not ensue. The effect of Ca^{2+} can also be seen in the DSC scan of TPE–CHEMS (92/8) (Figure 5f). In the presence of 3 mM Ca^{2+}, the hexagonal transition temperature of the lipid mixture decreased from 67 to 62 °C, which is still 6 °C above the H_{II} formation temperature of TPE.

Ca^{2+} caused similar changes to DOPE–CHEMS (7/3) vesicles: liposomes aggregated by 5 mM Ca^{2+} had a hexagonal transition temperature of 30–37 °C, almost 20 °C higher than that of pure DOPE (NMR spectra not shown).

DISCUSSION

Interactions of CHEMS with TPE at Neutral pH. Although the 3β-OH of CHEMS is esterified, CHEMS retains the affinity of cholesterol for phospholipids (Simmonds et al., 1984; Lai et al., 1984). The interaction of CHEMS with DMPE, leading to the broadening and eventual suppression of the main transition of DMPE, is indistinguishable from that of cholesterol (Lai et al., 1984). Similar results were demonstrated with TPE–CHEMS in this study. Owing to the heterogeneous acyl chain composition, the order–fluid transition of TPE proceeds in a less cooperative way than DMPE as indicated by the width of the main transition peak. A lower content of CHEMS (30 mol %) was enough to broaden the main transition of TPE so that is was not longer detectable by DSC.

PE vesicles are difficult to prepare at neutral pH. For PE with highly unsaturated acyl chains, the integrity of the bilayer surface cannot be maintained due to the H_{II} phase formation at a low temperature (Cullis & de Kruijff, 1979; Mantsch et al., 1981). For more saturated PEs which do not undergo the hexagonal transition until high temperature (Harlos & Eibl, 1981; Seddon et al., 1983), aggregates of lamellar structure are formed at pH 7.0 (Kolber & Haynes, 1979). These

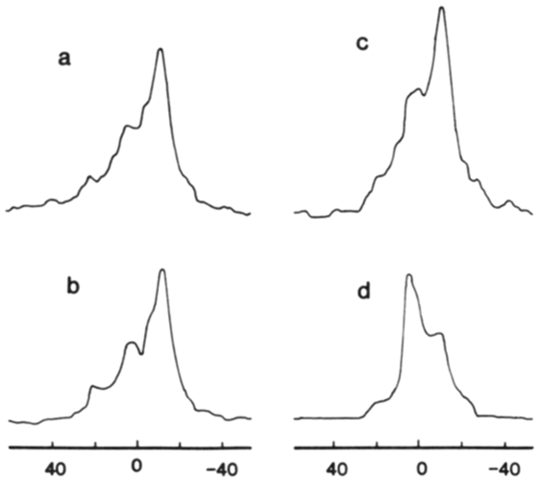

FIGURE 8: Proton-decoupled ^{31}P NMR spectra at 40.5 MHz of TPE–CHEMS (70/30) aggregated by dialysis against 3 mM Ca^{2+}, pH 7.4. (a) 40, (b) 55, (c) 66, and (d) 78 °C.

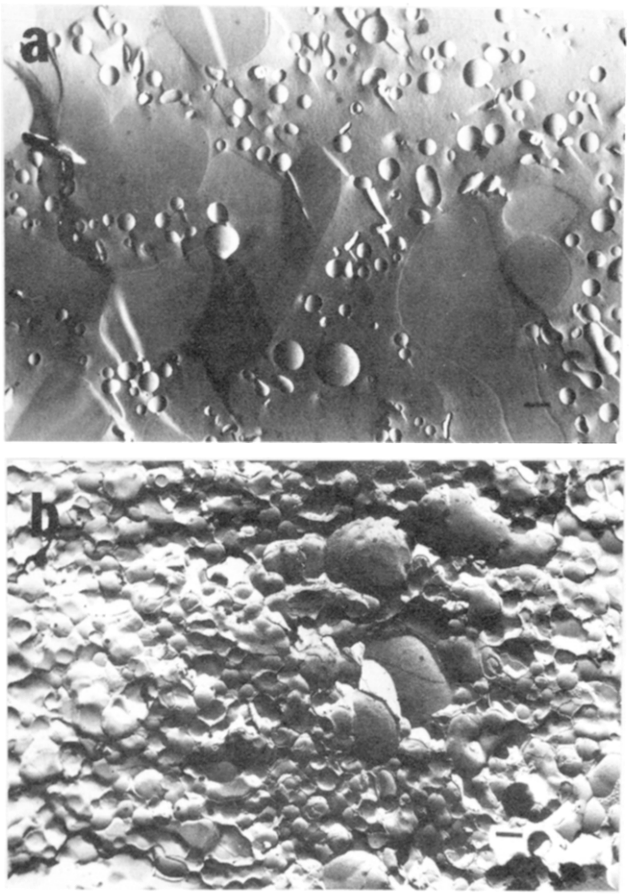

FIGURE 9: Freeze–fracture electron micrograph of TPE–CHEMS (70/30) (prepared by reversed-phase evaporation method, extruded through 0.2-μm polycarbonate membrane). (a) Before and (b) after the dialysis against 3 mM Ca^{2+} at 25 °C. Bar is 0.2 μm.

properties have been attributed to the conformation of the phosphoethanolamine head groups which allow a strong intermolecular interaction between the ammonium and phosphate groups of adjacent PEs (Hitchcock et al., 1974; Hauser et al., 1981). In the present study we have demonstrated that the incorporation of CHEMS stabilizes PE in bilayer membranes. The stabilization effect of CHEMS is manifested in both the inhibition of the lamellar to hexagonal phase transition and in the formation of bilayer vesicles of PE. The incorporation of 8 mol % CHEMS raises the L–H transition temperature of TPE by 11 °C (Figure 1b), whereas additional CHEMS moves the hexagonal transition beyond 72 °C (Figure 1c–e). When the mole ratio of CHEMS exceeds 20%, TPE forms stable vesicles. Similar results were observed for DMPE (Lai et al., 1984) and DOPE. The mole ratio of CHEMS required to stabilize PE in a bilayer is in the same range as that of PC on PE membranes (Hui et al., 1981; Tilcock et al., 1982; Dekker et al., 1983; Boni & Hui, 1983). The membrane-stabilization effect of CHEMS is probably due to the disruption of the intermolecular interaction between adjacent PEs, in a fashion similar to other phospholipids (Kolber & Haynes, 1979; Hui et al., 1981, Tilcock et al., 1982), or that observed when PE is deprotonated by raising the pH (Stollery & Vail, 1977).

Effect of pH on CHEMS–PE Mixtures. The protonation of CHEMS immediately leads to the aggregation of TPE–CHEMS and DOPE–CHEMS liposomes with the leaking of the encapsulated contents (Ellens et al., 1984a). Of particular interest is that the L–H transition is dramatically lowered, while the main transition of TPE–CHEMS is not affected by the protonation of CHEMS. At 30 mol % CHEMS, the order–fluid transition of TPE was suppressed at pH 4.5 (Figure 4e). Even after a 24-h incubation at pH 4.5 at a temperature above the L–H transition, the main transition of TPE was not detected. This implies that protonated CHEMS remains effectively complexed with phosphatidylethanolamine. CHEMS is, therefore, different from acidic phospholipids in that macroscopic lateral phase separation does not occur following the protonation of the charged group.

Lateral phase separation occurs when the clustering of each type of lipid is energetically favored over the mixture of the lipids (Trauble, 1976). The association of protonated CHEMS with TPE is, therefore, more stable than the segregation of CHEMS from TPE. We have shown that the 3β-OH of cholesterol is not required for the suppression of phospholipid order–fluid transition (Lai et al., 1985). Here, we have also demonstrated that phase transition suppression persists even after titration of the carboxylate group. It is therefore unlikely that the interaction of CHEMS and phospholipid involves hydrogen bonding between the carboxylate group of CHEMS and a polar component of phospholipid. Since the orientation of the sterol in the phospholipid membranes determines the extent of sterol–phospholipid interaction (Cadenhead & Muller-Landau, 1979; Lai et al., 1985), the results here suggest that the protonated CHEMS remains in a configuration that allows the maximum van der Waals interaction between sterol and phospholipid.

The hexagonal phase is adopted by a lipid when the area of the acyl chain region exceeds the head group area (Cullis & de Kruijff, 1979; Israelachvili et al., 1980; Mantsch et al., 1981). The association of column-shaped lipid with cholesterol has been suggested to produce a conically shaped molecule that prefers the hexagonal phase (Cullis & de Kruijff, 1979; Wieslander et al., 1980). We believe the tight association of CHEMS with TPE promotes the "conical-shaped" TPE complex as CHEMS becomes uncharged. It may be noted that the H_{II}-promoting effect of protonated CHEMS is unusually high compared to that of other sterols (Gallay & de Kruijff, 1982; Gallay et al., 1984). While the addition of 50 mol % cholesterol to PE decreases the hexagonal transition temperature by only 10–20 °C (Cullis & de Kruijff, 1978; Dekker et al., 1983), the incorporation of 25 mol % CHEMS reduced the L–H transition temperature of TPE by 30 °C at pH 4.5. Since the extent of interaction with phospholipid is almost the same for CHEMS and cholesterol (Lai et al., 1984; Simmonds et al., 1984), the greater H_{II}-promoting effect should be due to the presence of the succinyl ester. The exact molecular mechanism involved requires further elucidation.

The endothermic peak of the L–H transition of TPE is broadened by the addition of CHEMS. An interesting finding is that the acid-induced H_{II} endothermal peak is suppressed as the molar ratio of CHEMS in TPE exceeds 35% (Figure 4f) even though the ^{31}P NMR spectra indicate that a L–H transition still occurs (Figure 6f–h). This transition is observed in a temperature interval of 10–15 °C, implying that the hexagonal transition is still cooperative. The suppression of the endothermic H_{II} transition, therefore, is different from the suppression of the main transition of phospholipids by sterols. A possibility is that after sufficient amounts of CHEMS intercalate between PE molecules, the energy barrier between the lamellar and hexagonal phases of TPE–CHEMS becomes too small to be detected by calorimetry as CHEMS is titrated. This is also the case for PE with homogeneous unsaturated acyl chains such as DOPE, in which the L–H transition is not detected by DSC (van Dijck et al., 1976; Cullis & de Kruijff, 1979).

Effect of Calcium on CHEMS–PE Membranes. Both Ca^{2+} ions and protons induce the order–fluid transition of acidic phospholipids through charge neutralization (Trauble & Eibl, 1974; Jacobson & Papahadjopoulos, 1975; Trauble, 1976). Calcium ions also induce phase separation of bilayers containing PS and PA (Jacobson & Papahadjopoulos, 1975; van Dijck et al., 1978). While PE is only slightly affected by the presence of Ca^{2+} at neutral pH (Harlos & Eibl, 1980b), its mixture with CHEMS is very sensitive to Ca^{2+}. The TPE–CHEMS vesicles are readily aggregated upon the addition of calcium. The examination of lipid aggregates by EM showed fused structures that have a ^{31}P NMR characteristic of lamellar phase. NMR spectra indicated the L–H transition of Ca^{2+}–(TPE–CHEMS) occurs at a temperature higher than the H_{II} formation temperature of TPE (Figure 8). In contrast to the H_{II}-promoting effect of protons, Ca^{2+} seems to stabilize TPE in the lamellar phase when CHEMS is present. It is well-known that calcium ions and protons have different effects on the phase transition of acidic phospholipids. The addition of Ca^{2+} shifts the order–fluid transition to a much higher temperature than protonation (Jacobson & Papahadjopoulos, 1975; van Dijck, 1978; Liao & Prestegard, 1981). On the basis of these observations, Jacobson & Papahadjopoulos (1975) have suggested that the addition of Ca^{2+} to anionic phospholipids involves more than charge neutralization. X-ray diffraction indicates that calcium addition results in a close opposition of PS bilayers (Newton et al., 1978). Similar complexes of Ca^{2+}–PG (Harlos & Eibl, 1980a) and Ca^{2+}–PA (Liao & Prestegard, 1981) have also been characterized. Portis et al. (1979) proposed a trans complex between Ca^{2+} and the PS head group in which Ca^{2+} ions form a bridge between apposed bilayers. A study on the metastable phase transition of Ca^{2+}–PG led to a similar model (Boggs & Rangaraj, 1983). McLaughlin (1982) has demonstrated that the carboxylate group is the major site in PS where divalent cations form a tight complex. We propose a similar type of crystallization of CHEMS by Ca^{2+}. The rigid lattice of Ca^{2+}–CHEMS holds TPE in the lamellar phase even at temperatures above the hexagonal transition temperature of TPE. There is, however, one major difference between calcium–CHEMS and calcium–acidic phospholipid complexes: the complexation of CHEMS with Ca^{2+} does not induce macroscopic phase separation in TPE–CHEMS membrane systems as indicated by DSC scans. The observations that the charge neutralization by both H^+ and Ca^{2+} does not lead to extensive lateral phase separation reflects the more specific interaction between CHEMS and neutral phospholipids, which is lacking between acidic phospholipids and neutral phospholipids. Because of this strong association, titration of the succinyl group with protons results in the enhancement of H_{II} phase is TPE. On the other hand, when CHEMS is chelated by calcium to form a stable lamellar structure, the TPE is also immobilized in the lamellar phase.

Possible Biological Significance. The presence of H_{II} phase lipid has been implicated in several biological functions such as membrane fusion, transbilayer transport (Cullis & de Kruijff, 1979), and membrane enzyme activity (Madden et al., 1983). The presence of the acidic cholesterol ester inhibits the formation of the hexagonal phase and stabilizes PE in bilayer membranes at neutral pH. This might be the reason for the observed membrane stabilization effect of cholesteryl sulfate on erythrocytes and spermatozoa (Beau et al., 1974; Langlais, 1981) and for the pathological state in the skin of patients with recessive X-linked ichthyosis (Williams & Elias, 1981). We have demonstrated here that the properties of a model membrane can be regulated by charge neutralization. Similar effects could occur in biological membranes at high levels of calcium or by enzymatic removal of the sulfate or other groups. Although a direct relationship between biofunction and cholesteryl sulfate remains to be established, the present study provides a structural basis for how such effects could originate.

CONCLUSION

In the present study we have used cholesteryl hemisuccinate to demonstrate the unusual properties of anionic cholesterol esters. Charged cholesteryl hemisuccinate is different from cholesterol in its capacity to stabilize PE in bilayer membranes and to prevent the formation of the hexagonal phase. The acidic cholesterol ester is also different from acidic phospholipids due to its specific interaction with phospholipids and its suppression of the order–fluid transition of the associated phospholipids. Upon neutralization by protons or Ca^{2+}, cholesteryl hemisuccinate retains a substantial affinity for the phospholipid, and macroscopic phase separation does not ensue. The association of protonated cholesterol ester with phospholipid promotes the formation of a hexagonal phase, whereas the interaction of calcium-chelated CHEMS with PE may stabilize PE in a lamellar phase. These findings point out the possible biological relevance of acidic cholesteryl esters in biomembranes.

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REFERENCES

Bleau, G., Bodleg, F. H., Longpre, J., Chapdelaine, A., & Roberts, K. D. (1974) *Biochim. Biophys. Acta 352*, 1–9.

Boggs, J. M., & Rangaraj, B. (1983) *Biochemistry 22*, 5425–5435.

Boggs, J. M., Stamp, D., Hughes, D. W., & Deber, C. W. (1981) *Biochemistry 20*, 5728–5735.

Boni, L. T., & Hui, S. W. (1983) *Biochim. Biophys. Acta 131*, 177–185.

Candenhead, D. A., & Muller-Landau, F. (1979) *Chem. Phys. Lipids 25*, 329–343.

Colombat, A., Motta, C., Jouanel, P., Greil, J. D., Panouse-Perrin, J., Dastugue, B., & Delattre, J. (1981) *Biochimie 63*, 795–798.

Cullis, P. R., & de Kruijff, B. (1978) *Biochim. Biophys. Acta 507*, 207–218.

Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.

Dekker, C. J., Geurts van Kessel, W. S. M., Klomp, J. P. G., Pieters, J., & de Kruijff, B. (1983) *Chem. Phys. Lipids 33*, 93–106.

Ellens, H., Bentz, J., & Szoka, F. C. (1984a) *Biochemistry 23*, 1532–1538.

Ellens, H., Bentz, J., & Szoka, F. C. (1984b) *Biophys. J. 45*, 70a.

Galla, H.-J., & Sackmann, E. (1975) *J. Am. Chem. Soc. 97*, 4114–4120.

Gallay, J., & de Kruijff, B. (1982) *FEBS Lett. 143*, 133–136.

Gallay, J., de Kruijff, B., & Demel, R. A. (1984) *Biochim. Biophys. Acta 769*, 96–104.

Harlos, K., & Eibl, H. (1980a) *Biochemistry 19*, 895–899.

Harlos, K., & Eibl, H. (1980b) *Biochim. Biophys. Acta 601*, 113–122.

Harlos, K., & Eibl, H. (1981) *Biochemistry 21*, 2888–2892.

Hauser, H., Finer, E. G., & Darke, A. (1977) *Biochem. Biophys. Res. Commun. 76*, 267–274.

Hauser, H., Pascher, I., Pearson, R. H., & Sundell, S. (1981) *Biochim. Biophys. Acta 650*, 21–51.

Hitchcock, P. B., Mason, R., Thomas, K. M., & Shipley, G. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3036–3040.

Hui, S. W., Stewart, T. P., Yeagle, P. L., & Albert, A. D. (1981) *Arch. Biochem. Biophys. 207*, 227–240.

Israelachvili, J. N., Marceljia, S., & Horn, R. G. (1980) *Q. Rev. Biophys. 13*, 121–200.

Jacobson, K., & Papahadjopoulos, D. (1975) *Biochemistry 14*, 152–161.

Kolber, M. A., & Haynes, D. H. (1979) *J. Membr. Biol. 48*, 95–114.

Lai, M.-Z., Szoka, F. C., & Düzgüneş, N. (1984) *Biophys. J. 45*, 192a.

Lai, M.-Z., Dügüneş, N., & Szoka, F. C. (1985) *Biochemistry* (preceding paper in this issue).

Langlais, J., Zolliger, M., Plamte, L., Chapdelaine, A., Bleau, G., & Roberts, K. D. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7266–7280.

Liao, M. J., & Prestegard, J. H. (1981) *Biochim. Biophys. Acta 645*, 149–156.

Litman, B. J. (1973) *Biochemistry 12*, 2545–2554.

Madden, T. D., Hope, M. J., & Cullis, P. R. (1983) *Biochemistry 22*, 1970–1974.

Mantsch, H. H., Martin, A., & Cameron, D. G. (1981) *Biochemistry 20*, 3138–3145.

McLaughlin, A. C. (1982) *Biochemistry 21*, 4879–4885.

Moser, H. W., Moser, A. B., & Orr, J. (1966) *Biochim. Biophys. Acta 116*, 146–155.

Newton, C., Pangborn, W., Nir, S., & Papahadjopoulos, D. (1978) *Biochim. Biophys. Acta 506*, 281–287.

Papahadjopoulos, D., & Miller, N. (1967) *Biochim. Biophys. Acta 135*, 624–628.

Papahadjopoulos, D., Poste, G., Schaeffer, B. E., & Vasil, W. J. (1974) *Biochim. Biophys. Acta 352*, 10–28.

Papahadjopoulos, D., Vail, W. J., Pangborn, W. A., & Poste, G. (1976) *Biochim. Biophys. Acta 448*, 265–283.

Papahadjopoulos, D., Vail, W. J., Newton, C., Nir, S., Jacobson, K., Poste, G., & Lazo, R. (1977) *Biochim. Biophys. Acta 465*, 579–598.

Pascher, I., & Sundell, S. (1977) *Acta. Chem. Scand. A31*, 767–774.

Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780–790.

Seddon, J. M., Ceve, G., & Marsh, D. (1983) *Biochemistry 22*, 1280–1289.

Simmonds, A. C., Rooney, E. K., & Lee, A. G. (1984) *Biochemistry 23*, 1432–1441.

Stollery, J. G., & Vail, W. J. (1977) *Biochim. Biophys. Acta 471*, 372–390.

Tilcock, C. P. S., Balley, M. B., Farem, S. B., & Cullis, P. R. (1982) *Biochemistry 21*, 4596–4601.

Tokutomi, S., Ohki, K., & Ohnishi, S. I. (1980) *Biochim. Biophys. Acta 596*, 192–200.

Trauble, H. (1976) in *Structure of Biological Membranes* (Abrahamsson, S., & Pascher, I. Eds.) pp 509–550, Plenum Press, New York.

Trauble, H., & Eibl, H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 214–219.

Vail, J., Stollery, J. G. (1978) *J. Microsc. (Oxford) 113*, 107–108.

van Dijck, P. W. N., de Kruijff, B., van Deenen, L. L. M., de Gier, J., & Demel, R. A. (1976) *Biochim. Biophys. Acta 455*, 576–587.

van Dijck, P. W. N., de Kruijff, B., Verkleij, A. J., van Deenen, L. L. M., & de Gier, J. (1978) *Biochim. Biophys. Acta 512*, 84–96.

Verkleij, A. J., & de Gier, J. (1981) in *Liposomes: From Physical Structure to Therapeutic Applications* (Knight, C. G., Ed.) pp 83–103, Elsevier/North-Holland Biomedical Press, Amsterdam.

Weislander, A., Cristiansson, A., Rilfors, L., & Lindblom, G. (1980) *Biochemistry 19*, 3650–3655.

Williams, M. L., & Elias, P. M. (1981) *J. Clin. Invest. 68*, 1404–1410.