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Anomalous mixing of zwitterionic and anionic phospholipids with double-chain cationic amphiphiles in lipid bilayers

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High-sensitivity scanning calorimetry has been used to examine the thermotropic behavior of mixtures combining dipalmitoylphosphatidylcholine (DPPC), phosphatidylethanolamine (DPPE) and *O*-methylphosphatidic acid (DPPA-OMe) with the double-chain cationic amphiphiles *N,N*-dihexadecyl-*N,N*-dimethylammonium chloride (DHDAC), 1,2-dipalmitoyloxy-3-(trimethylammonio)propane (DPTAP) and the corresponding monomethylated tertiary amino compounds (DHMMA-H$^+$ and DPDAP-H$^+$). At physiological ionic strength, mixtures of these cationic amphiphiles with the anionic phospholipid DPPA-OMe can show gel-to-liquid-crystalline phase transitions at considerably higher temperatures than do either of the pure components. Surprisingly, binary mixtures of DPPC and these cationic amphiphiles also show strongly nonideal mixing, with phase diagrams exhibiting pronounced maxima in their solidus and liquidus curves. Similar behavior is not observed for mixtures of DPPC with DPPA-OMe, which closely resembles DPTAP and DPDAP-H$^+$ in backbone configuration and polar headgroup size. The present results suggest that perturbation of the orientation of the phosphatidylcholine headgroup by cationic amphiphiles, as demonstrated previously by Seelig and co-workers (Biochemistry 28 [1989], 7720–7728), can significantly affect the thermotropic behavior of phospholipids such as DPPC. Such effects may exert a generally important (though not always easily recognizable) influence on the organization and thermotropic behavior of systems where zwitterionic phospholipids are combined with charged bilayer-associated molecules.

Introduction

The physical properties of mixtures of neutral phospholipids and charged amphiphiles are of considerable interest to model the behavior of the lipid component of biological membranes. While a number of studies have examined the thermotropic behavior of binary mixtures of neutral and anionic phospholipids [1–15], few have examined the behavior of codispersions of phospholipids with cationic amphiphiles [16–21] and particularly with cationic amphiphiles that, like natural membrane lipids, spontaneously form bilayers in aqueous dispersion [22–24]. Such phospholipid/cationic amphiphile dispersions show distinctive abilities to interact efficiently with biological membranes and to deliver associated bioactive molecules to eukaryotic cells [25–31], and elucidation of their physical properties may further our general understanding of the interactions between neutral phospholipids and charged amphiphilic components of biological membranes.

In this study, differential scanning calorimetry has been used to examine the intermixing of dipalmitoyl phospholipids (PC, PE and *O*-methyl-phosphatidic acid) with different cationic tertiary and quaternary amine amphiphiles carrying two sixteen-carbon hydrocarbon chains. The binary lipid systems described here were initially of interest because they offer the opportunity to compare the mixing of phospholipids with different bilayer-forming amphiphiles that have closely related structures but qualitatively distinct hydrogen-bonding abilities. While the different lipid mixtures examined show no differences in behavior that can be

Abbreviations: DHDAC, *N,N*-dihexadecyl-*N,N*-dimethylammonium chloride; DHMMA, *N,N*-dihexadecyl-*N*-monomethylamine; DPDAP, 1,2-dipalmitoyloxy-3-dimethylaminopropane; DPPA-OMe, 1,2-dipalmitoyl-*O*-methylphosphatidic acid; (DP)PC, (1,2-dipalmitoyl)phosphatidylcholine; (DP)PE, (1,2-dipalmitoyl)phosphatidylethanolamine; DPTAP, 1,2-dipalmitoyloxy-3-(trimethylammonio)propane.

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clearly attributed to hydrogen-bonding effects, all show strongly nonideal mixing of the phospholipid and the cationic-amphiphile component, such that mixtures of these species often give higher transition temperatures than does either of the pure components. These results suggest that electrostatic interactions exert significant effects on the packing and the thermotropic behavior of zwitterionic phospholipids in these mixtures, and by implication in other systems where zwitterionic phospholipids interact with charged lipids or other charged amphiphiles associated with the lipid bilayer.

Materials and Methods

Materials

Dipalmitoyl phosphatidylcholine (99 + % purity) was obtained from Sigma (St.-Louis, MO) and used as received. All fatty acids and long-chain alkyl methanesulfonates were purchased from Nu-Chek Prep (Elysian, MN). All other chemicals were of at least reagent grade; all solvents were HPLC grade and/or were redistilled before use. Dipalmitoyl-*O*-methylphosphatidic acid was prepared from dipalmitoylphosphatidylcholine by phospholipase D-mediated transphosphatidylation in the presence of 10% methanol as described by Comfurius and Zwaal [32]. DPDAP was prepared by acylation of 3-dimethylamino-1,2-propanediol with palmitoyl chloride, and a portion was converted to DPTAP by reaction with methyl iodide, as described previously for the corresponding dioleoyl compounds [30].

N,N-Dihexadecyl-N,N-dimethylammonium chloride. Dimethylhexadecylamine, prepared by overnight reaction of hexadecyl methanesulfonate (150 mg) at 65°C with 4 ml of 30% methanolic dimethylamine, was heated for 36 h at 65°C with 200 mg of hexadecyl methanesulfonate in 4 ml of 1:1 chloroform/acetonitrile. The products of this reaction were partitioned between chloroform and 1:1 methanol/1 M aqueous KCl, and the chloroform phase was washed six times with equal volumes of the same methanol/aqueous mixture to convert the ammonium compound to its chloride form. After concentration of the chloroform phase in vacuo, the products were purified by 'flash' chromatography [33] on a 13 × 2 cm column of Bio-Sil A, eluting with ten column volumes each of 0%, 3% and 5% methanol in chloroform to yield 170 mg of the desired product in pure form at 5% methanol. The structure of the product was confirmed by proton NMR.

N,N-Dimethyl-N-methylamine. 500 mg of hexadecyl mesylate was reacted overnight with 3 ml 30% aqueous methylamine and 6 ml ethanol. After evaporation of the solvents under nitrogen, the products were partitioned between chloroform and 1:1 methanol/0.1 M aqueous NaOH, and the chloroform phase was twice washed with the same mixture and then concentrated in vacuo. To the thoroughly dried products (377 mg) was added an equal weight of hexadecyl mesylate in 5 ml of 1:1 chloroform/acetonitrile, and the mixture was heated to 65°C for 36 h under nitrogen. After evaporation of the solvents, the products of the above reaction were reacted with an excess of acetic anhydride (0.3 ml) plus triethylamine (0.2 ml) in 3 ml chloroform for 2 h at 25°C. The products from this reaction (the desired trialkylamine, plus lesser amounts of tetraalkylammonium and acetylated dialkylamine compounds) were purified by chromatography on a column of BioSil A, eluting with a gradient of 0–4% methanol in chloroform (plus 0.05% triethylamine) to yield pure dihexadecylmethylamine at 4% methanol. The structure of the product was confirmed by proton NMR.

Methods

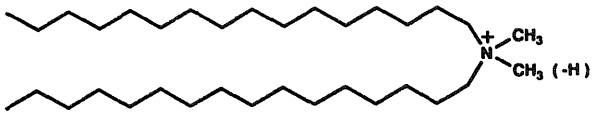
Phospholipids for the preparation of stock solutions were standardized by phosphorus assay as described elsewhere [34], while cationic amphiphiles were standardized by drying to constant weight from acetone or cyclohexane under high vacuum in the presence of P_2O_5. Lipid samples for calorimetry were dried down from chloroform, then colyophilized from 97:3 cyclohexane/ethanol. The dried samples were redispersed by vortexing in buffer solutions containing 150 mM NaCl plus 50 mM sodium acetate (for pH ≤ 5.6) or 50 mM glycine (for pH ≥ 8.5), heated briefly above the transition temperature, then cooled at approx. 1 C°/min to 4°C and finally incubated at the latter temperature for 4 days (at pH ≤ 3.5) or 7 days (at pH ≥ 4.5). Samples of pure DPPC prepared in this manner at pH 4.5 or 9.5 showed no detectable hydrolysis products when subsequently extracted and analyzed by thin-layer chromatography (0.5 μmol lipid analyzed). DPPC samples prepared in this manner at pH 3.0 showed a small amount of degradation (1–3% based on lyso-PC formed) which was negligible in samples containing 20% or more cationic amphiphile.

Samples prepared as described above were analyzed by high-sensitivity differential scanning calorimetry, and phase diagrams were deduced from the thermograms for samples of varying composition, as described previously [13,35]. Sample masses for transition enthalpy determinations were determined from the results of phosphorus analysis [34] and the known proportions of phospholipid in the samples.

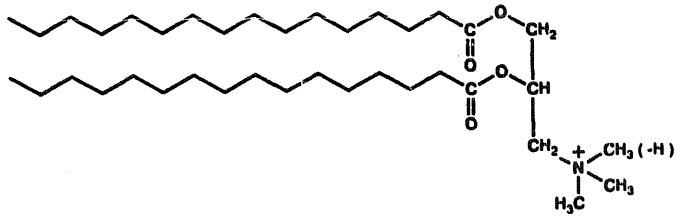
Results

The structures of the quaternary and tertiary amine amphiphiles prepared in this study, and for comparison those of the dipalmitoyl phospholipids examined, are shown in Fig. 1. In preliminary experiments, the calorimetric behavior of dispersions of the different amine

DHDAC (DHMMA)



DPTAP (DPDAP)



DPP-X

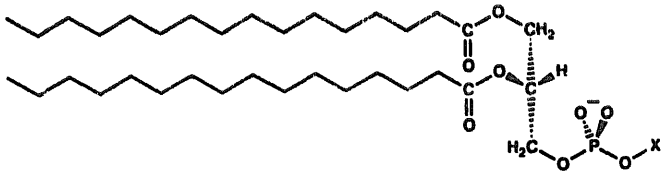


Fig. 1. Structures and abbreviations of the quaternary and tertiary amine amphiphiles prepared in this study, and of a 1-α-1,2-dipalmitoyl phospholipid.

amphiphiles was examined at physiological ionic strength and various pH values. As illustrated in Fig. 2, at pH 3.0–9.5 dispersions of *N,N*-dihexadecyl-*N,N*-dimethylammonium chloride (DHDAC) incubated for several hours to days at 4°C exhibit a major transition centered at 36.8°C, accompanied by a low-temperature shoulder and smaller lower-temperature transitions. DHDAC dispersions that were rapidly cooled from the liquid-crystalline state to ≤ 20°C just before beginning the calorimetric scan showed a different, less highly endothermic transition at 30.7°C (not shown), indicating the existence of a second, metastable gel state. Accurate determination of molar enthalpies of transition was difficult for pure dispersions of this and the other cationic amphiphiles examined, as sample aggregation complicated attempts to introduce a precisely measured amount of the amphiphile into the calorimeter cells. However, for dispersions of DHDAC the molar enthalpies of the major transitions could be roughly estimated as approx. 15.6 kcal mol^{-1} (for the stable solid phase) and 7.1 kcal mol^{-1} (for the metastable solid phase), respectively. At pH ≤ 3.0, dispersions of DHMMA-H^+, the monomethylated tertiary amine analogue of DHDAC, show a major transition at 65.7°C, with some heat evolution at lower temperatures indicating metastable behavior. At alkaline pH, pure DHMMA formed highly floculent dispersions which were unsuitable for calorimetric analysis. 1,2-Dipalmitoyloxy-3-dimethylaminopropane (DPDAP) gives a transition centered at 56.2°C when dispersed at pH ≤ 3.5, while the trimethylammonio analogue DPTAP gives a major transition centered at 49.3°C at pH 3.0–5.6.

While the thermotropic properties of aqueous dispersions of the bromide salts of DHDAC and DPTAP have been described previously [36–38], these results cannot be directly compared to those found here due to significant differences in the ionic strength, counterions and methods of sample preparation used in the different studies. Nonetheless, the above results are broadly consistent with those reported previously for these species.

In Fig. 3 are shown heating thermograms for representative mixtures of DPPC and DHDAC dispersed in 150 mM NaCl, 50 mM sodium acetate (pH 4.5) and in Fig. 4A is shown the phase diagram deduced for this system. Very similar results were obtained at pH 3.0

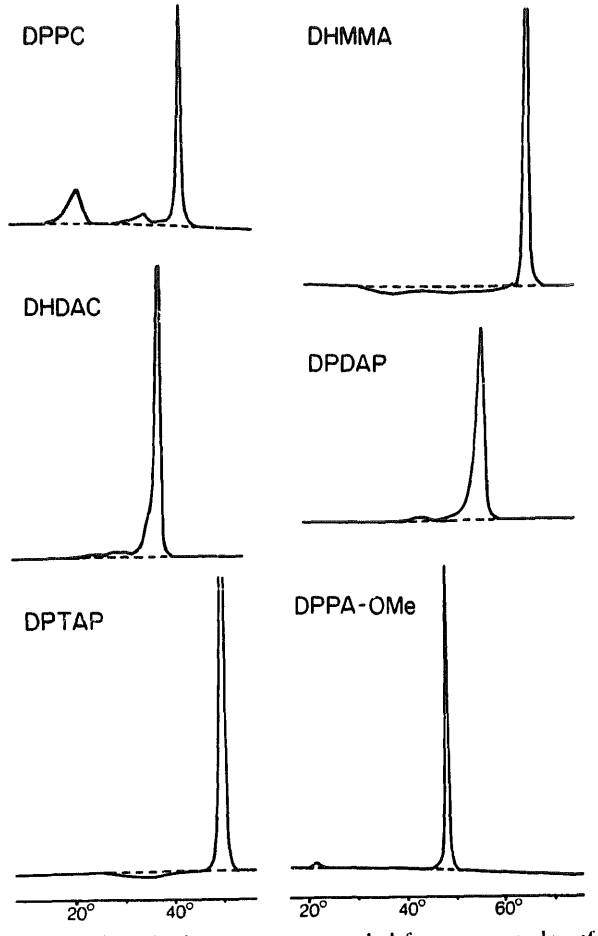


Fig. 2. Calorimetric thermograms recorded for pure samples of the phospholipids and cationic amphiphiles examined in this study. Samples were prepared and equilibrated in 150 mM NaCl, 50 mM sodium acetate as described in Materials and Methods. Traces shown for DPPC, DHDAC and DPTAP were obtained at pH 4.5; very similar traces were obtained at pH 3.0 or 9.5. Traces shown for DHMMA-H^+ and DPDAP-H^+ were obtained at pH 3.0 and are identical to those obtained at pH 2.5. The thermogram shown for DPPA-OMe was obtained at pH 5.6. The major transitions for some thermograms are plotted off-scale to show smaller transitions clearly.

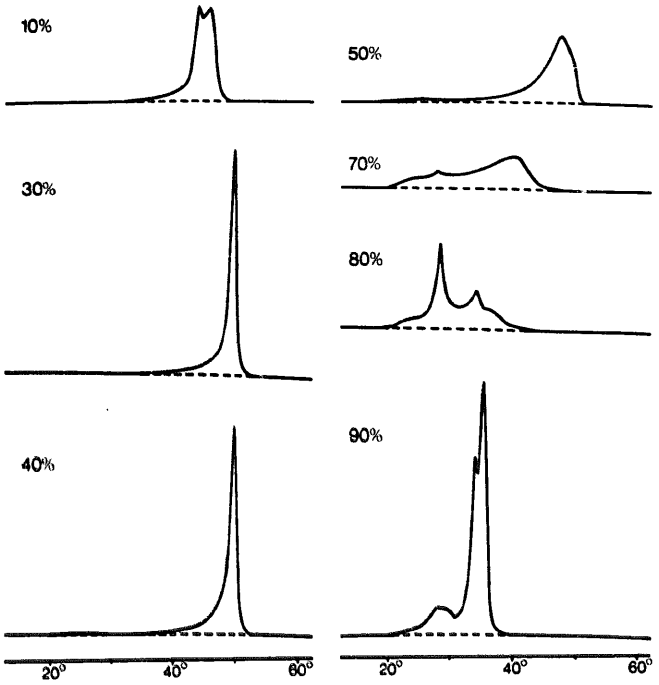


Fig. 3. Calorimetric thermograms obtained for DPPC/DHDAC mixtures containing the indicated mole percentages of DHDAC. Samples were prepared at pH 4.5, and calorimetric analysis was carried out, as described in Materials and Methods. The thermogram for pure DPPC at this pH is shown in Fig. 2.

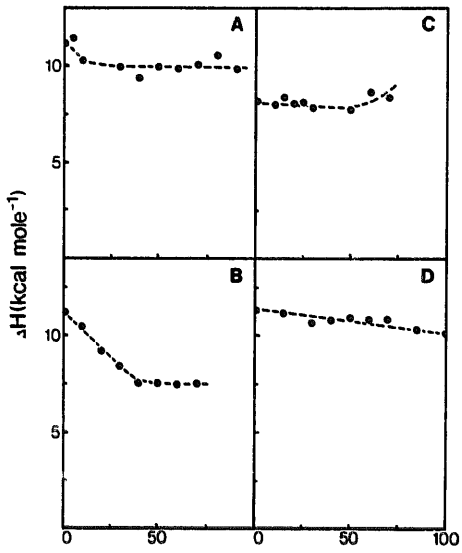


Fig. 5. Total enthalpies of the main plus (where present) pre-transitions for mixtures of (A) DPPC and DHDAC at pH 4.5; (B) DPPC and DHMMA-H$^+$ at pH 3.0; (C) DPPE and DHDAC at pH 4.5; and (D) DPPC and DPPA-OMe at pH 5.6. Details of sample preparation and determination of transition enthalpies are given in Materials and Methods.

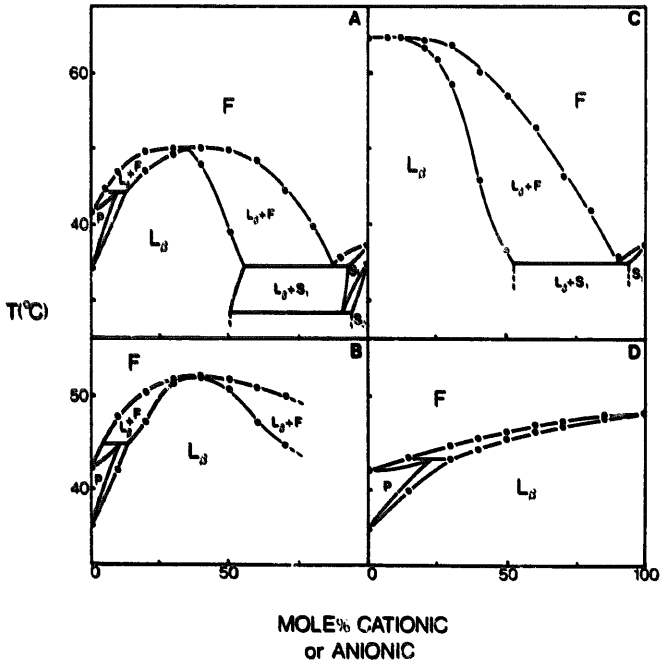


Fig. 4. Calorimetrically derived phase diagrams for the systems (A) DPPC/DHDAC at pH 4.5; (B) DPPC/DHMMA-H$^+$ at pH 3.0; (C) DPPE/DHDAC at pH 4.5; and (D) DPPC/DPPA-OMe at pH 5.6. Details of sample preparation and calorimetric analysis were as described in Materials and Methods. Phases are represented as follows in the figure: F = fluid (liquid-crystalline lamellar); $L_\beta = L_\beta$ or $L_{\beta'}$ gel phases; P = $P_{\beta'}$ 'ripple' phase of DPPC; S_1 and S_2, solid phases rich in DHDAC. The right-hand side of panels A and B represents 100 mol% cationic amphiphile; this label is omitted from the x-axis for clarity.

(not shown). Two points are most striking: first, the main transition temperature is higher for most DPPC/DHDAC mixtures than for either of the pure components, reaching a maximum temperature of 50.0°C at approx. 35 mol% DHDAC, and second, the sharpness of the main transition at this composition approaches that measured for the pure components. These features are reflected in the phase diagram for this system as a convergence of the solidus and liquidus curves to a clear maximum at approx. 35 mol% DHDAC (Fig. 4A). At high mole fractions of DHDAC, solid-solid phase separation is observed at low temperatures. The total enthalpies of the $L_{\beta'}$-to-liquid-crystalline transitions for various DPPC/DHDAC mixtures are plotted as a function of composition in Fig. 5A. No clear maximum or other noteworthy variation in the transition enthalpy is observed in the range of compositions bracketing the maximum in the phase diagram for this system.

In Fig. 4B is shown the calorimetrically derived phase diagram obtained for the system DPPC/DHMMA-H$^+$ at pH 3.0. The behavior of this system is qualitatively very similar to that for the DPPC-DHDAC system discussed above, although samples containing very high mole fractions ($\geq$ 80 mol%) of DHMMA-H$^+$ are prone to metastable behavior, complicating the correct determination of the phase diagram for this range of compositions. Samples containing lower mole fractions of DHMMA-H$^+$ did not show metastable behavior and gave very similar thermograms whether

preincubated at 2°C for 7 days or for 6 h after initial dispersal. Like the DPPC-DHDAC system, the DPPC/DHMMA-H^+ system shows a pronounced maximum at approx. 40 mol% DHMMA-H^+. The temperature of this maximum is only slightly higher than for the DPPC/DHDAC system (51.9°C vs. 50.0°C), and again, the sample composition giving a maximum transition temperature also gives a transition width comparable to that for either of the pure components. As shown in Fig. 5B, the total enthalpy for the $L_{\beta'}$-to-liquid-crystalline transition of different DPPC/DHMMA-H^+ mixtures falls with increasing content of DHMMA-H^+ up to approx. 40 mol%, then levels off at higher mole fractions of the cationic amphiphile. Again, the transition enthalpy shows no discernible maximum in the range of compositions around the maximum observed in the phase diagram for this system.

Thermograms for DPPC/DHMMA mixtures containing ≥ 20 mol% DHMMA at pH ≥ 9.0 exhibited two resolved endothermic components, which varied in relative magnitude but not in either position or shape as the proportion of DHMMA was varied above 20 mol% (not shown). This behavior indicates that the neutral form of DHMMA exhibits only limited solubility in either gel- or liquid-crystalline DPPC bilayers, in contrast to the behavior of the protonated form.

In Fig. 4C is shown the calorimetrically derived phase diagram for the DPPE/DHDAC system. No outright maximum is observed in the phase diagram for this system. However, as indicated, mixtures containing up to 15 mol% DHDAC exhibit a phase transition essentially identical in temperature and sharpness to that observed for pure DPPE samples, while mixtures with higher DHDAC contents show substantially broader phase transitions and lower transition temperatures. This behavior suggests that the DPPE/DHDAC system shows a deviation from ideal behavior (which can be expressed quantitatively as described in the Appendix) that is qualitatively similar to that for the DPPC/DHDAC and DPPC/DHMMA-H^+ systems but which is considerably smaller in magnitude. The enthalpy of the main transition for different DPPE/DHDAC mixtures shows little variation with composition up to at least 50 mol% DHDAC; strong aggregation hindered quantitative recovery of samples (and hence precise determination of sample masses and molar transition enthalpies) for mixtures with high DHDAC contents.

As the thermograms shown in Fig. 6 illustrate, other mixtures of dipalmitoyl phospholipids and di-C_{16} cationic amphiphiles also exhibit phase transitions at temperatures well above those of the individual pure components. At pH 5.6 or 4.5, an equimolar mixture of DPPC and DPTAP shows a sharp phase transition centered at 56.4°C, while the individual components

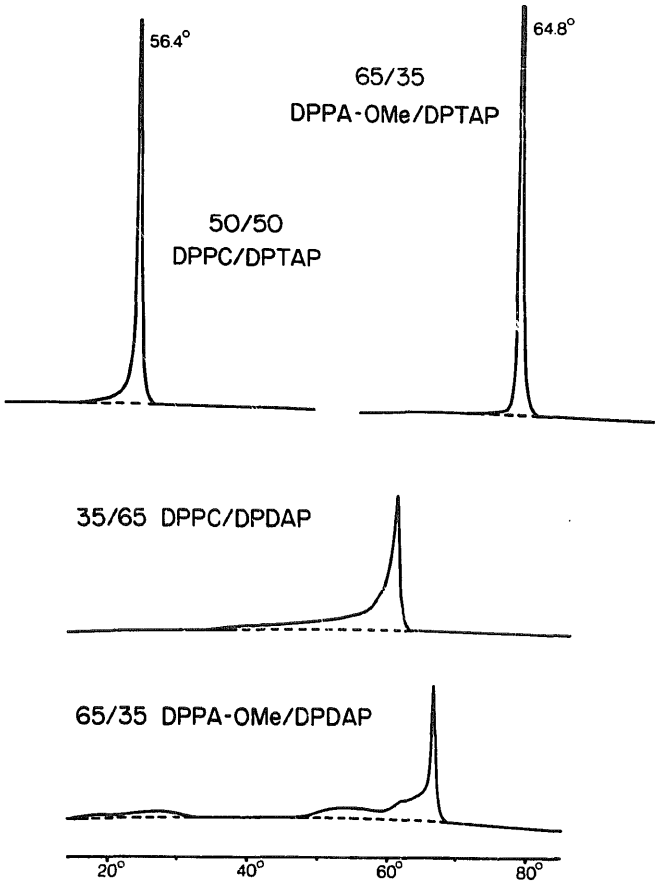


Fig. 6. Calorimetric thermograms for mixtures of phospholipids and cationic amphiphiles of the indicated compositions. The temperature scale for the lower two traces is indicated at the bottom of the figure; the upper two traces are shifted but are plotted with the same spacing on the temperature axis. Thermograms shown were recorded for samples prepared at the following pH values: 4.5 for DPPC/DPTAP, 3.0 for DPPC/DPDAP and 5.6 for DPPA-OMe/DPTAP and DPPA-OMe/DPDAP.

show transitions at 41.9°C and 49.3°C, respectively (Fig. 2). A 65:35 mixture of DPDAP-H^+ (T_c = 56.2°C) and DPPC (T_c = 41.7°C) at pH 3.0 shows a broader, asymmetrical transition peaking at 61.2°C. Even more dramatic increases in the main transition temperature are observed when DPTAP or DPDAP-H^+ is combined with the anionic phospholipid DPPA-OMe. A 65:35 DPPA-OMe/DPTAP mixture at pH 5.6 or 4.5 shows a sharp phase transition at 64.8°C, well above the phase transition temperatures of the two components (48.0°C and 49.3°C, respectively). Similarly, a 65:35 mixture of DPPA-OMe (T_c = 48.0°C) and DPDAP-H^+ (T_c = 56.2°C) at these same pH values shows a complex melting profile that extends up to a peak at 66.5°C. Mixtures of DPPA-OMe with DHDAC also gave broad phase transitions extending up to 57.8°C (not shown), well above the transition temperatures of the pure components (48.0°C and 36.8°C, respectively).

A final series of experiments examined the mixing of DPPC with DPPA-OMe, an anionic species which is similar in overall geometry and headgroup size to DP-

TAP and DPDAP-H^+. Mixtures of DPPC and DPPA-OMe in all proportions give narrow phase transitions at temperatures intermediate between the transition temperatures of the two components. The calorimetrically derived phase diagram for this system (Fig. 4D) is qualitatively different from those observed for the DPPC/cationic amphiphile mixtures discussed above. While the presence of multiple low-temperature phases for DPPC complicates attempts to analyze the phase diagram quantitatively, the mixing of DPPC and DPPA-OMe appears almost ideal. The total enthalpy of the $L_{\beta'}$-to-liquid-crystalline phase transition for these mixtures shows an essentially linear variation with composition between the values measured for the pure components (Fig. 5D).

Discussion

The most striking feature of the behavior of the phospholipid/cationic amphiphile systems studied here is the fact that almost all of them exhibit higher phase transition temperatures for mixed-lipid samples than for samples of their pure components. Such behavior has been observed before for certain mixtures of phospholipids with single-chain amphiphiles [18–21,39–44] but has rarely been observed in mixtures such as those studied here, where both of the components are double-chain, bilayer-forming amphiphiles [22].

The observation of an elevation of the main transition temperature for mixtures of double-chain cationic amphiphiles with the anionic phospholipid DPPA-OMe is perhaps not surprising. Similar (but less dramatic) elevations of the transition temperature have been observed before for mixtures of anionic phospholipids with single-chain cationic amphiphiles [21], and Eibl and Wooley [22] have reported that an equimolar mixture of dimyristoyl-*O*-methylphosphatidic acid (DMPA-OMe) and 1,2-dimyristoyloxy-3-dimethylaminopropane-H^+ (DMDAP-H^+) shows a considerably higher transition temperature than do the pure components. However, these latter workers reported that an equimolar mixture of DMPA-OMe and 1,2-dimyristoyloxy-3-(trimethylammonio)propane (DMTAP) showed a comparatively small elevation of the transition temperature, and they therefore ascribed the marked elevation of the transition for DMPA-OMe/DMDAP-H^+ mixtures primarily to intermolecular ($\gtrsim$N–H$\cdots$O=P$\lesssim$) hydrogen-bonding rather than to electrostatic effects. By contrast, our results indicate that quaternary amine amphiphiles such as DPTAP and DHDAC interact with DPPA-OMe in a similar manner as do the cognate tertiary amine amphiphiles DPDAP-H^+ and DHMMA-H^+, suggesting that the elevation of the main phase transition in these mixtures rests largely on electrostatic rather than hydrogen-bonding interactions. It is not clear why the dimyristoyl lipid mixtures studied by Eibl and Woolley [22] showed qualitatively different behavior than the cognate dipalmitoyl lipid mixtures studied here, although differences in conditions of sample preparation and ionic strength employed in the two studies may be at least partly responsible.

More surprising than the above results is the observation that mixtures of DPPC with several different cationic dihexadecyl or dipalmitoyl amphiphiles also show main phase transitions at substantially higher temperatures than do the pure components. This behavior apparently does not depend on highly specific features of the cationic amphiphile's headgroup or backbone structure but does require the presence of the positive charge; neither the anionic species DPPA-OMe (which resembles DPDAP-H^+ and DPTAP in its backbone configuration and headgroup size) nor the unprotonated form of DHMMA mix with DPPC in this distinctive manner. It may seem surprising that the elevation of the main transition temperature for mixtures of DPPC with cationic species such as DHDAC and DHMMA-H^+ is not accompanied by a substantial increase in the transition enthalpy. However, even using regular solution theory, which assumes that nonidealities in mixing are attributable entirely to enthalpic effects, we can calculate that the transition enthalpies for the DPPC/DHDAC and DPPC/DHMMA-H^+ mixtures giving maximum transition temperatures should exceed by only a few hundred cal mol^{-1} (see Appendix) the values expected if lipid mixing was ideal. Even smaller elevations of the transition enthalpy may be expected for these mixtures if the components also exhibit nonzero excess *entropies* of mixing. The transition-enthalpy data shown in Figs. 5A and 5B are consistent with these expectations.

A potential explanation for the behavior of the DPPC/cationic amphiphile systems discussed above is offered by the finding [24] that the introduction of increasing amounts of cationic amphiphiles into PC bilayers causes a reorientation of the PC phosphocholine moiety, progressively increasing the average angle of the P-to-N axis with respect to the bilayer plane. In the systems examined here, such a reorientation could decrease the effective cross-sectional area of the PC headgroup by modifying steric and/or electrostatic (dipolar) interactions between headgroups [44,45], allowing tighter intermolecular packing in mixed DPPC/cationic amphiphile bilayers than is found in bilayers of pure DPPC. At the same time, intermolecular interactions may be stronger in the DPPC/cationic-amphiphile mixtures than in bilayers containing purely the cationic species, as mixing of PC with the cationic amphiphile will decrease the electrostatic free energy of the latter both by simple dilution of the

surface charge [46,47] and possibly by favorable interactions between the positive charges and the PC headgroup dipoles. These effects are expected to be more important in the gel state, where the mean molecular area is smaller and steric and/or electrostatic repulsions between headgroups should be greater, than in the liquid-crystalline state. Taking these factors together, we can rationalize the finding that the phase diagrams for mixtures of DPPC with cationic amphiphiles such as DHDAC exhibit maxima in their liquidus and solidus curves. Similar behavior is not expected (and is not observed) for mixtures combining DPPC with anionic phospholipids such as DPPA-OMe (this paper) or DPPG [6], which cause an opposite shift in the orientation of the phosphatidylcholine P-to-N axis with respect to the bilayer plane [48–51].

The hypothesis described above is also compatible with the finding that the phase diagram for the DPPE/DHDAC system shows clearly nonideal mixing but not a true maximum in the solidus and liquidus curves. It is possible that this result implies simply that bilayer-intercalated cationic amphiphiles cause a smaller reorientation of the PE headgroup than of the PC headgroup. A more probable explanation for the different behavior of the DPPC/DHDAC and of the DPPE/DHDAC systems is that the DPPE headgroup is already 'small' enough, even in its normal orientation, that shifts in its orientation induced by cationic amphiphiles such as DHDAC would have comparatively small effects on intermolecular packing. This latter possibility is quite reasonable given that saturated PEs occupy significantly smaller molecular areas in gel-state bilayers than do saturated PCs [52–57].

The above interpretations, while they are consistent with both the present and previous experimental findings, may describe only approximately the physical basis for the distinctive thermotropic behavior of the zwitterionic phospholipid/cationic amphiphile mixtures examined here. The present results nonetheless strongly suggest that the incorporation of charged molecules into a membrane containing zwitterionic phospholipids can alter the thermotropic behavior of the latter species through electrostatic interactions between the zwitterionic lipid headgroups and the introduced surface charges. Such effects are probably also at work (but may be more difficult to identify unambiguously) in other systems where zwitterionic phospholipids are combined with charged bilayer-intercalated molecules, including charged lipids, proteins and polypeptides, and drugs [44,50,58–68]. Failure to consider the existence of such effects in these systems may lead to erroneous conclusions concerning the physical mechanisms by which bilayer-intercalated charged molecules influence the thermotropic behavior (and, by inference, the molecular organization) of phospholipid bilayers.

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Appendix

Prediction of the excess enthalpy of the gel-to-liquid-crystalline transition for an 'azeotropic' lipid mixture from regular solution theory

The chemical potentials for the components in a lipid mixture with coexisting gel and liquid-crystalline phases can be expressed [58] as

$$\mu_i^{l} = \mu_i^{o,l} + RT(\ln x_i^{l}) + \mu_i^{e,l}$$

$$\mu_i^{g} = \mu_i^{o,g} + RT(\ln x_i^{g}) + \mu_i^{e,g}$$

where x_i is the mole fraction of the ith component, the superscripts ^{g} and ^{l} refer to the gel and to the liquid-crystalline states, respectively, and the superscripts ^{o} and ^{e} refer to the standard chemical potential and to the excess molar free energy of mixing for each component. In regular solution theory, the excess molar free energy of mixing is assumed to be completely enthalpic in nature and is represented by the term h_i^{e}.

For a binary system whose phase diagram exhibits a maximum at a temperature T_{max} and composition x_i^{max}, at the point of intersection of the solidus and liquidus curves $x_i^{g} = x_i^{l}$ and $\mu_i^{g} = \mu_i^{l}$. Combining these restrictions with the above equations and using the above assumption that $\mu_i^{e} = h_i^{e}$, at the temperature and composition of the maximum in the phase diagram

$$(\mu_i^{o,l} - \mu_i^{o,g}) + (h_i^{e,l} - h_i^{e,g}) = \Delta H_i^{o} - T_{max}\Delta S_i^{o} + \Delta h_i^{e} = 0$$

(assuming that the heat capacity changes slowly with temperature outside the phase transition region), and hence

$$\Delta h_i^{e} = \Delta H_i^{o}([T_{max}/T_i^{o}] - 1)$$

where ΔH_i^{o} and ΔS_s^{o}solid phase of i represent the standard enthalpies and entropies of transition of the components, T_i^{o} is the transition temperature of pure component i and $\Delta h_i^{e} = (h_i^{e,l} - h_i^{e,g})$.

The excess enthalpy for the gel-to-liquid crystalline phase transition at the composition corresponding to the maximum in a binary phase diagram such as those shown in Figs. 4A or 4B will thus be given by

$$\Delta h_{total}^{e} = \Delta h_A^{e} + \Delta h_B^{e}$$

$$= x_A^{max}(\Delta H_A^{o}[(T_{max}/T_A^{o}) - 1])$$

$$+ (1 - x_A^{max})(\Delta H_B^{o}[(T_{max}/T_B^{o}) - 1])$$

For the DPPC/DHDAC system, the choice of appropriate values of T_0 and ΔH_B^o to use in the above calculation is not obvious, as the equilbrium low-temperature solid phase of pure DHDAC is different from the $L_{\beta'}$ phase formed by DPPC and by DPPC/DHDAC mixtures of the composition around the maximum in the phase diagram. To calculate reasonable limits for Δh^e_{total}, however, we can use the transition temperatures and enthalpy values measured for the metastable and for the stable 'gel' phases of DHDAC (see Results). Using either of these sets of values together with the corresponding. parameters for DPPC, we obtain similar estimates of Δh^e_{total}, namely 350 cal mol^{-1} and 420 cal mol^{-1}.

A similar analysis can be applied to the phase diagram for the system DPPE/DHDAC (Fig. 4C) if we assume that this phase diagram exhibits a very weak maximum at approx. 10 mol% DHDAC. While the transition temperature for this composition is at best marginally higher than that for pure DPPE, the transition is essentially identical in width to that for DPPE and is markedly narrower (and occurs at a markedly higher temperature) than that expected even for ideal mixing of the two components. Under this assumption we estimate a value for Δh^e_{total} of 75–130 cal mol^{-1} at the putative 'maximum' point in the DPPE/DHDAC phase diagram, again depending on which transition of pure DHDAC is used to estimate the transition temperature and enthalpy for this component.

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