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Spin-Labeling Study of Phosphatidylcholine–Cardiolipin Binary Mixtures†

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ABSTRACT: Electron paramagnetic resonance spectra of the spin-label probe 2,2,6,6-tetramethylpiperidinyl-1-oxy have been used to study the phase behavior of binary mixtures of different phosphatidylcholines (dipalmitoyl, distearoyl, and dioleoyl) with cardiolipin, using either calcium-free or calcium-containing cardiolipin (with a calcium:cardiolipin ratio of 1:2) samples. Results show that the nature of the fatty acid chains of the phosphatidylcholines (chain length and unsaturation) may influence the coexistence of different phases as well as does the nature of the cation linked to the cardiolipin.

Binary mixtures of lipids have been extensively investigated by many different techniques, with the aim of finding a correlation between composition and structure for these mixtures. One of the main goals has been to find whether different phases, and in particular immiscible fluid phases, may exist in the plane of a phospholipid bilayer. Such lipid phase separations are biologically significant: (i) they affect the lateral molecular motion and therefore are involved in immune processes (McConnell, 1978); (ii) by modifying the lateral compressibility of the membrane, they play an important role in the insertion of proteins (Shimshick & McConnell, 1973); and (iii) by changing the state of the lipidic environment around a protein, they modulate the enzymatic activity (Wisnieski et al., 1974). In addition, calcium-induced phase modifications can be involved in transport phenomena and fusion processes (Verkleij et al., 1979).

One example of the immiscibility of lipid phases is the dimyristoylphosphatidylcholine (DMPC)[1]–cholesterol mixture for which a great number of techniques or approaches have been used, including lateral diffusion coefficient measurements (Rubenstein et al., 1979), calorimetry (Melchior & Steim, 1979), freeze–fracture electron microscopy (Copeland & McConnell, 1980), and spin-labeling studies (Rubenstein et al., 1979; Recktenwald & McConnell, 1981). Several attempts have been made to discuss the properties of such binary mixtures in terms of phase diagrams (Shimshick & McConnell, 1973; Lee, 1977; Lentz et al., 1980).

A second example is the binary mixture of DMPC and cardiolipin. For this system, spin-labeling and lateral diffusion

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; CL, cardiolipin; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; EPR, electron paramagnetic resonance.

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coefficient measurements led to the conclusion that immiscible fluid phases are present in the lamellar lipid mixture (Berclaz & McConnell, 1981). Since many important properties of membrane systems are related to the fatty acid chain of respectively the host matrix and the added phospholipid, we decided to investigate if the previously observed phase behavior for DMPC–cardiolipin mixtures is affected by the variation in the fatty acid chain length of the host matrix.

We have therefore investigated the thermodynamic modifications induced by the addition of increasing amounts of cardiolipin into binary mixtures of cardiolipin with phosphatidylcholines—dipalmitoyl (DPPC), distearoyl (DSPC), and dioleoyl (DOPC). By looking at the partition of the spin-label probe 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo) between the aqueous and the lipidic phases of such binary mixtures, we endeavor to answer the following questions: (i) Is the coexistence of phases into liposomes made with cardiolipin and phosphatidylcholine dependent upon the nature of the phosphatidylcholine? (ii) Does the cation associated with the cardiolipin molecule have any influence on the observed thermodynamic behavior of such binary mixtures?

Materials and Methods

The phosphatidylcholines DPPC, DSPC, and DOPC were purchased from Sigma Chemical Co. (St Louis, MO) and stored at 17 mM stock solutions in MeOH–$CHCl_3$ (50:50) under argon. They were used without further purification.

Bovine heart cardiolipin was purchased (a) from Sigma Chemical Co. (St Louis, MO) as 3.34 or 4.0 mg/mL in EtOH. Supplied as the sodium salt, this cardiolipin was shown by atom absorption spectrophotometry to be a mixture of sodium and calcium salts. Purification was undertaken with the following experimental procedure [adapted from Shimojo & Ohno (1966)]: cardiolipin solution in MeOH was evaporated to dryness and then dissolved, under argon, in $CHCl_3$ to a final concentration of around 15 mg/mL. Such a solution was then applied to a column packed with approximately 10 g of silicic acid (100 mesh, Mallinckrodt) and eluted with 100-mL $CHCl_3$:MeOH mixtures with ratios of 98:2, 95:5, 90:10, 80:20, and 50:50. Fractions of about 10 mL were collected at a flow rate of 2–3 mL/min. Thin-layer chromatography revealed two well-separated populations corresponding to the calcium salt (eluted first) and sodium salt cardiolipin (eluted last). The two fractions were dried and carefully weighed and kept as 2.2 mM stock solutions in MeOH–$CHCl_3$ (50:50) mixtures under argon. Bovine heart cardiolipin was also purchased (b) from Supelco Inc. (Bellefonte, PA) as 50 mg/mL in $CHCl_3$ under argon. After chromatography, this cardiolipin showed only one fraction corresponding to the calcium salt. Tempo was used at ca. 10^{-4} M in phosphate-buffered saline (PBS) solutions containing 1 mM ethylenediaminetetraacetic acid (EDTA).

Sample Preparation. For the measurements of Tempo partition coefficients, liposomes were prepared by following a previously described procedure (Rubenstein et al., 1980). The hydration of the lipidic films was undertaken at 10 °C above the phase transition temperature of the pure phosphatidylcholines for DPPC and DSPC and at room temperature for the DOPC.

EPR spectra were taken, using 50- and 100-μL sealed capillary tubes, with a Varian E-9 EPR spectrometer according to a previously described procedure (Rubenstein et al., 1980). The temperature was measured with a Pt sensor and a MEIER TMK-2 device.

Tempo partition into phosphatidylcholine–cardiolipin liposomes was measured by monitoring the high-field EPR signal

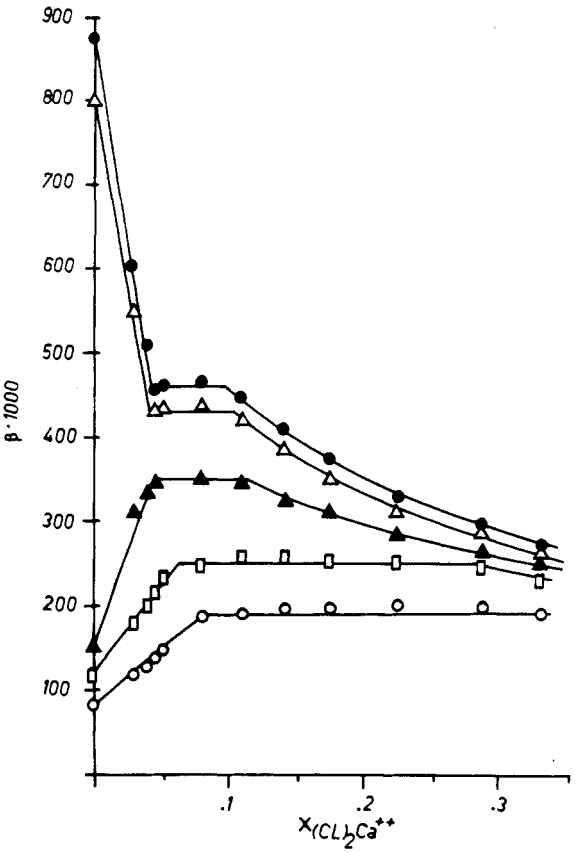

FIGURE 1: Tempo partition coefficient β into DPPC–cardiolipin liposomes as a function of the mole fraction of CL_2Ca^{2+} at (○) 32, (□) 36, (▲) 40, (△) 46, and (●) 50 °C.

for Tempo dissolved in the hydrophobic (H) and polar (P) environments. The partition coefficient β (given by the ratio H/P of the two signals) was calculated on a Hewlett-Packard HP 9830 desk computer with the help of a Summagraphics digitalizing table. Electron micrographs have been obtained with a Zeiss EM 109 instrument (magnification 4400×) by using the negative (phosphotungstate) staining technique (Munn, 1974).

Results

Figure 1 shows the Tempo partition coefficient β as a function of the mole fraction of CL_2Ca^{2+}, and Figure 2 shows the partition data corresponding to the cardiolipin containing only Na^+ ions (and no Ca^{2+}), both Figures referring to the DPPC–cardiolipin binary mixtures.

The data in Figures 3 and 4 refer to the binary mixtures of DSPC and cardiolipin, using cardiolipin samples containing Ca^{2+} ions (Figure 3) or Na^+ ions (Figure 4).

The data in Figures 5 and 6 refer to Ca^{2+}–cardiolipin (Figure 5) and Na^+-cardiolipin (Figure 6) systems for binary mixtures of DOPC and cardiolipin.

The experimental points have been measured at constant lipid weight (5 mg) at different temperatures. Due to a lack of reproducibility of some experimental points, results for mole fractions of cardiolipin higher than 0.5 are not shown.

In Figures 1–6, a number of experimental points have been omitted to avoid overcrowding. All the paramagnetic resonance spectra show well-resolved high-field hydrophobic and polar signals. The accuracy of the experimental values is of the order of ±3%.

Note that the partition coefficients have been measured in temperature ranges including the gel–liquid–crystalline phase transition of the pure host matrix in the case of DPPC and

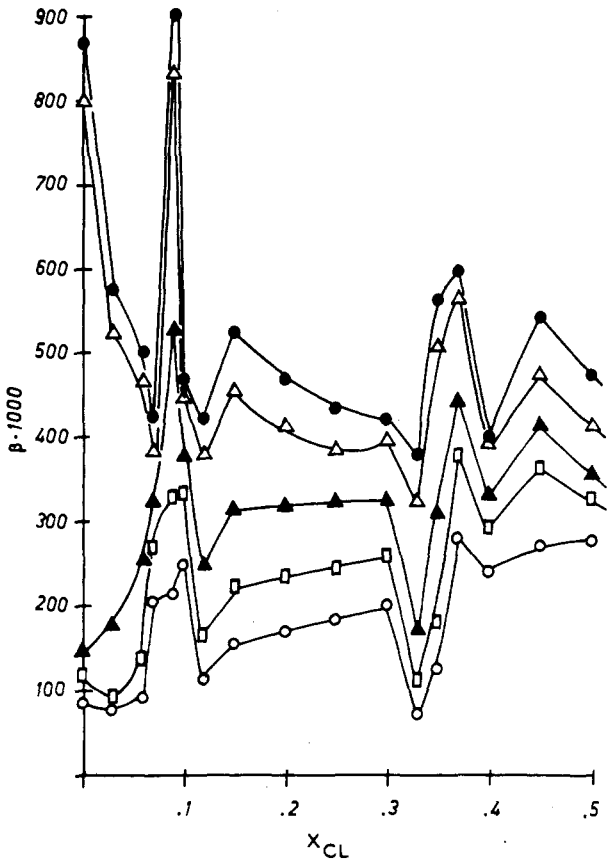

FIGURE 2: Tempo partition coefficient β into DPPC–cardiolipin liposomes as a function of the mole fraction of CL for the calcium-free cardiolipin sample at (○) 32, (□) 36, (▲) 40, (△) 46, and (●) 50 °C.

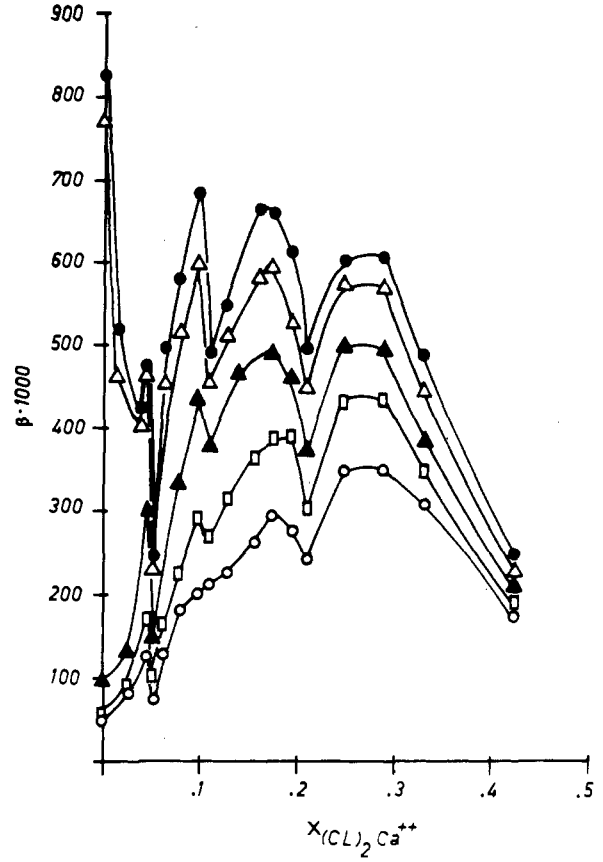

FIGURE 3: Tempo partition coefficient β into DSPC–cardiolipin liposomes as a function of the mole fraction of CL_2Ca^{2+} at (○) 42, (□) 46, (▲) 50, (△) 56, and (●) 60 °C.

DSPC (Figures 1–4) but well above such a phase transition for DOPC (Figures 5 and 6). This explains the difference in the sign of $\delta\beta/\delta X$ for the highest and lowest temperatures in Figures 1–4, for mole fractions of cardiolipin below 0.05, whereas this effect is not observed in Figures 5 and 6.

Figures 1 and 5 are the only ones in which the lines represent calculated values of the partition coefficient β (from the equation given under Discussion); in all other figures, the lines are only interpolations between data obtained at the same temperature.

To check possible macroscopic changes in the form of the sample, electron micrographs were obtained in the following cases: (a) for $X_{CL_2Ca^{2+}} = 0.02, 0.041, 0.085, 0.175$, and 0.275; (b) for $X_{CL} = 0.08, 0.09, 0.10, 0.11, 0.12$, and 0.15 in binary mixtures of DPPC with cardiolipin. Although these samples exhibit clear discontinuities in $\delta\beta/\delta X$ in these ranges of cardiolipin concentration, no change is observed in the corresponding micrographs.

Discussion

Considering the data in Figure 1 referring to the DPPC–(calcium–cardiolipin) mixtures, we find the same pattern as in our previous study of phase equilibria in binary mixtures of DMPC with cardiolipin. The discontinuities in $\delta\beta/\delta X$ must be phase boundaries, which, according to the phase rule, separate one- and two-phase regions for such binary mixtures (Berclaz & McConnell, 1981). As was shown for the DMPC–cardiolipin mixture, it is possible to write the equation for a binary system (of components A and B), giving the Tempo partition coefficient (β) in a two-phase system as a function of the Tempo partition coefficients β_i and β_j in the two phases i and j. If we take into account that the total weight of lipid is constant and that the lever rule allows one to obtain the fraction of all the molecules which are in a given phase, then the following equation gives the variation of β as a function of the mole fraction of one component (X_B) in a two-phase region, the limits of which are defined by the experimental values X_B^i, X_B^j and β_i, β_j:

$$\beta = \beta_j + (\beta_i - \beta_j)\frac{X_B^j - X_B}{X_B^j - X_B^i}\frac{(K-1)X_B^i + 1}{(K-1)X_B + 1}$$

where β_i and β_j are the partition coefficients of the phases i and j, respectively, X_B^i and X_B^j are the mole fractions of B in phases i and j, respectively, X_B is the mole fraction of B in the sample, and $K = M_r(A)/M_r(B)$ is the ratio of the molecular weights of the two components (A and B) of our binary mixture. For the DPPC–(calcium–cardiolipin) mixtures, it was only possible to fit the experimental values with the calculated ones if we assumed that cardiolipin was present as the calcium-bridged dimer CL_2Ca^{2+}, which was done in the calculation of the lines in Figure 1. This confirmed the previous observations concerning DMPC–cardiolipin mixtures (Berclaz & McConnell, 1981).

The Tempo partition data in Figure 5 (referring to DOPC–cardiolipin mixtures) have a marked extremum for $X_{CL_2Ca^{2+}} = 0.015$ which can be related to compound or eutectic formation. From that value of $X_{CL_2Ca^{2+}}$, the variation of β is analogous to that observed and described above for DPPC–cardiolipin mixtures. Here also, the calculated values could fit the experimental ones only if we assume the presence of the calcium-bridged dimer CL_2Ca^{2+}.

The Tempo partition data in Figures 2, 4, and 6, with their sharp maxima and minima, are similar to and as unusual as the data we have previously reported for binary mixtures containing DMPC and calcium-free cardiolipin samples, and

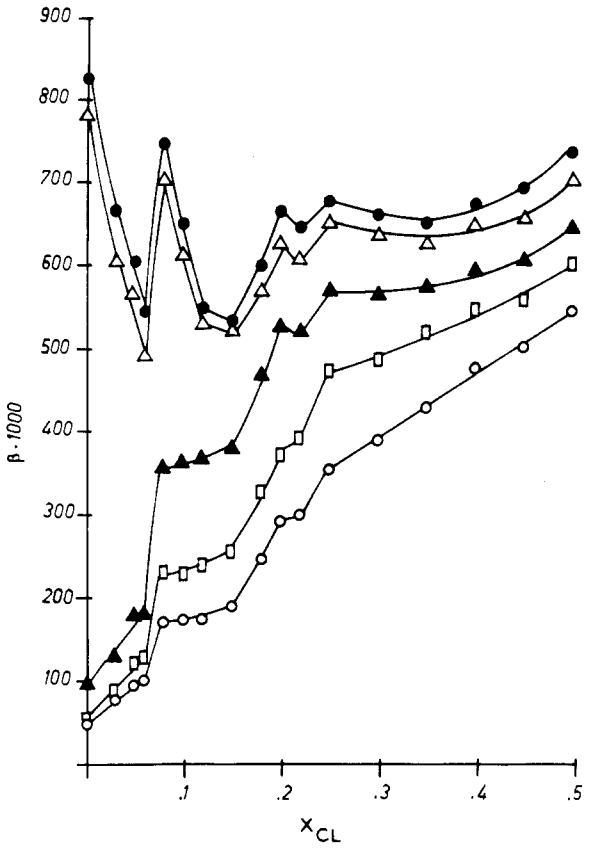

FIGURE 4: Tempo partition coefficient β into DSPC–cardiolipin liposomes as a function of the mole fraction of CL for the calcium-free cardiolipin sample at (○) 42, (□) 46, (▲) 50, (△) 56, and (●) 60 °C.

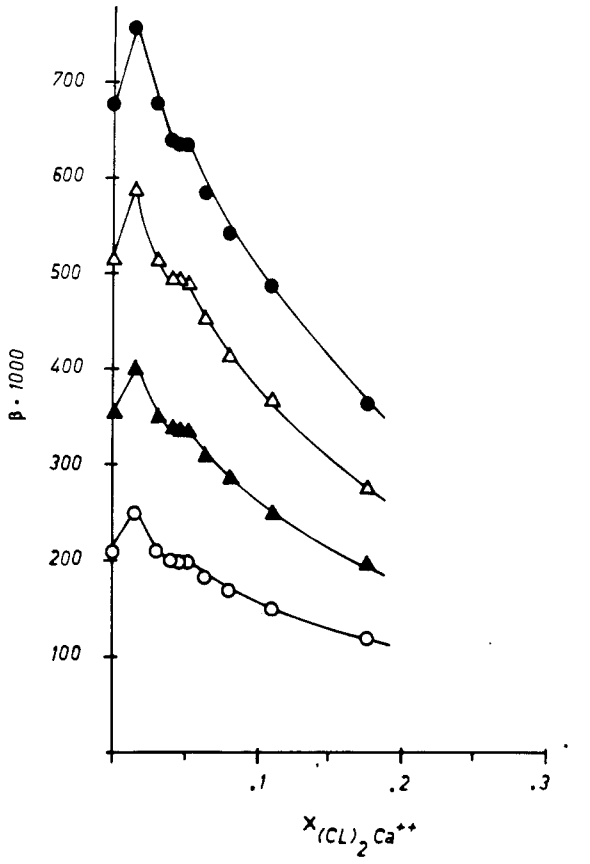

FIGURE 5: Tempo partition coefficient β into DOPC–cardiolipin liposomes as a function of the mole fraction of CL_2Ca^{2+} at (○) 10, (▲) 20, (△) 30, and (●) 40 °C.

may probably be related to compound formation.

If we consider the data in Figure 3 referring to the binary mixtures of DSPC with cardiolipin, we can easily see a marked difference in the behavior of DSPC with respect to that observed previously (for DMPC) and in this work (for DOPC and DPPC). Indeed, the simple discontinuities in $\delta\beta/\delta X$ have been replaced by sharp maxima and minima which have never been observed before with binary mixtures containing phosphatidylcholines and calcium salt cardiolipin samples. The results we obtain for DSPC indicate that, in this case, the marked discrepancies usually seen between binary mixtures having calcium-containing and calcium-free cardiolipin are not observed.

The reversibility of the temperature response has been investigated, for the two systems DPPC–CL_2Ca^{2+} and DPPC–CL, by the following procedure: for a given concentration of cardiolipin, the partition coefficient is successively measured at 36, 50, and 36 °C; the same measurements are repeated for various cardiolipin contents [$X_{CL_2Ca^{2+}}$ = 0.02, 0.035, 0.085, and 0.175 and X_{CL} = 0.06, 0.10, 0.15, and 0.37]. Within experimental precision, the β values do not show hysteresis.

From the electron micrographs, it appears that the discontinuities in $\delta\beta/\delta X$ are not due to macroscopic alteration of the liposomes; this is supported by the thermal reversibility of the partition coefficient.

It has already been shown that the acyl chain length can have an important effect on the thermodynamic properties of phospholipid preparations, such as the permeability of liposomes (de Gier et al., 1968; Block et al., 1975) as well as the fluidity and gel–liquid-crystalline phase transition characteristics for binary mixtures of phosphatidylcholines (Lentz et al., 1976). A similar dependence can also be expected for systems with cardiolipin as one component of a biological

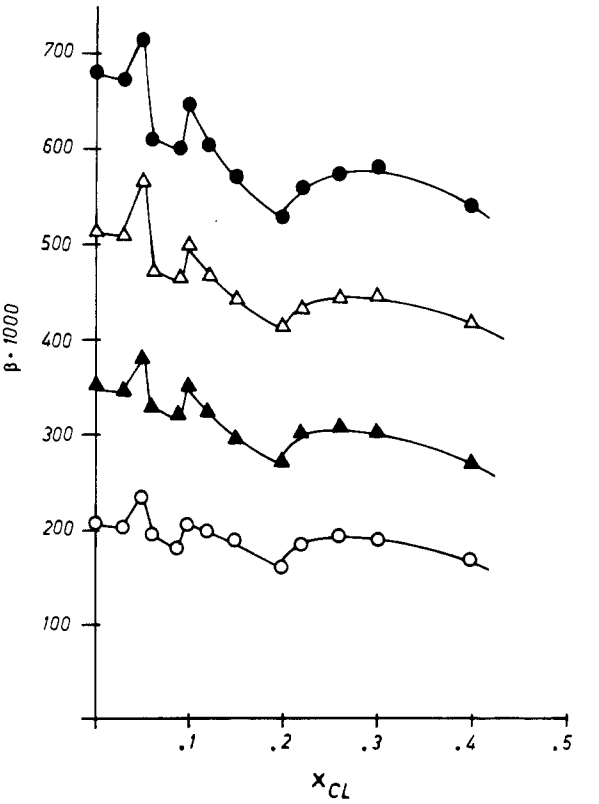

FIGURE 6: Tempo partition coefficient β into DOPC–cardiolipin liposomes as a function of the mole fraction of CL for the calcium-free cardiolipin sample at (○) 10, (▲) 20, (△) 30, and (●) 40 °C.

membrane. As the concentration of bovine heart cardiolipin lies in the range of 2–25%, spin-labeling is appropriate to the

study of such a dependence since this technique is particularly sensitive to small amounts of cardiolipin.

Our results show that the exact nature of the phosphatidylcholine can play an important role in the thermodynamic behavior of phospholipid liposomes made with cardiolipin. We cannot yet account for all the data in terms of phase diagrams. However, we can still conclude that immiscible fluid phases are present in binary mixtures of phosphatidylcholines and cardiolipin, that the acyl chain length of the host matrix can play a significant role in the phase behavior of these mixtures, and that the cation associated with the cardiolipin molecule also influences the thermodynamic behavior of such binary mixtures even though the nature of the cation (Na^+ or Ca^{2+}) cannot by itself allow us to predict such a behavior without taking into account the exact nature (acyl chain length and unsaturation) of the phosphatidylcholines.

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Registry No. DPPC, 2644-64-6; DSPC, 4539-70-2; DOPC, 10015-85-7; Ca, 7440-70-2; Na, 7440-23-5.

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