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A phase behavior study of mixtures of sphingosine with zwitterionic phospholipids

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Abstract

The interactions of sphingosine (SPH) with dipalmitoylphosphatidylcholine (DPPC) and dielaidoylphosphatidylethanolamine (DEPE) have been studied by means of differential scanning calorimetry (DSC) and ^{31}P-nuclear magnetic resonance (^{31}P-NMR). Experiments were carried out with the fully protonated form of SPH, at pH 6.0. DSC studies showed that the main T_c transition temperature of DPPC was perturbed by the presence of SPH so that T_c of the mixture was higher than those of pure components at concentrations of SPH up to 50 mol%, with an azeotropic point at 30 mol% of SPH. At higher concentrations solid phase separations were observed from 70 to 95 mol% of SPH with an eutectic point at 90 mol% of SPH. ^{31}P-NMR showed lamellar phases in DPPC/SPH mixtures, at all the range of concentrations. The behavior of DEPE/SPH mixtures was somewhat different since no azeotropic point was detected, the gel to liquid-crystalline transition being depressed by the presence of SPH, and an eutectic point was detected at 60 mol%. Solid phase immiscibilities were present between 50 mol% and 85 mol% of SPH. It is also remarkable that the liquid-crystalline to hexagonal H_{II} phase transition of DEPE was only slightly shifted to lower temperatures at concentrations of SPH lower than 33 mol% of SPH but, this transition disappeared at concentrations of SPH higher than 33 mol% of SPH, so that isotropic phases were formed instead, as seen through ^{31}P-NMR. The present results show the importance of taking into account the effects appearing in mixtures of SPH with zwitterionic phospholipids when considering their influence on the organization of biomembranes.

Key words: Sphingosine; Phosphatidylcholine; Phosphatidylethanolamine; DSC; NMR, ^{31}P-

1. Introduction

While a number of studies have examined the thermotropic behavior of binary mixtures of neutral and anionic phospholipids [1–4], few have examined the behavior of dispersions of phospholipids with cationic amphiphiles [5–10] and particularly sphingosine (SPH), an important biological mediator that affects many cellular functions [11,12]. SPH appears to be involved in the inhibition of tissue factor [13], inhibition of insulin receptor tyrosine kinase [14], biphasic effects on diacylglycerol kinase [15], inhibition of phosphatidic acid phosphohydrolase [16,17], inhibition of CTP-phosphocholine cytidyltransferase [18] and activation of phospholipase C [19,20]. Apart from that, SPH has been recognized as an inhibitor of protein kinase C [21] and this activity appears to mediate many biological effects of SPH [11,12]. Because of its ability to inhibit protein kinase C, SPH has been described as a cancer preventive agent [22]. It is clear that SPH must be preferentially partitioned into the membrane and despite its remarkable importance, in health and disease, very little is known about the behavior of SPH in membranes and its interaction with other lipids.

In this work we present the detailed study of the interaction of SPH with dipalmitoylphosphadityl-choline (DPPC) and dielaidoylphosphatidylethanol-

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPH, sphingosine; DPPC, *sn*-1,2-dipalmitoylphosphatidylcholine; DEPE, *sn*-1,2-dielaidoylphosphatidylethanolamine; ΔH, enthalpy change of the gel to liquid-crystalline phase transition; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ppm, parts per million; $\Delta\sigma$, chemical shift anisotropy; T_c, onset temperature of the gel to liquid-crystalline phase transition; T_H, onset temperature of the bilayer to hexagonal H_{II} phase transition ($L_\alpha \rightarrow H_{II}$).

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amine (DEPE) in order to understand the mechanism of action of this bioactive molecule, which is localized in cell membranes. Differential scanning calorimetry (DSC) and ^{31}P-nuclear magnetic resonance (^{31}P-NMR) have been used, showing non-ideal mixing of the mixtures in both cases. It should be remarked the formation of an azeotropic mixture at approx. 30 mol% of SPH when mixed with DPPC and an eutectic point at approx. 90 mol% of SPH. On the other hand, the mixtures DEPE/SPH showed an eutectic point at approx. 60 mol% of SPH and solid-solid phase separation at concentrations higher than 40 mol% of SPH. These results suggest that SPH exerts significant effects on the packing and the thermotropic behavior of zwitterionic phospholipids.

2. Materials and methods

sn-1,2-Dipalmitoylphosphatidylcholine (DPPC) and *sn*-1,2-dielaidoylphosphatidylethanolamine (DEPE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). D-Sphingosine (SPH) (from bovine brain sphingomyelin) was from Sigma (Madrid, Spain). Water was twice distilled and deionized in a Milli-Q apparatus from Millipore. All other reagents were of analytical grade. The purity of DEPE, DPPC and SPH was checked by thin-layer chromatography before and after the measurements, specially those experiments done at high temperature, where they showed only one spot in all cases.

2.1. Sample preparation

DEPE or DPPC (4 μmol for DSC and 40 μmol for ^{31}P-NMR experiments) in chloroform/methanol (2:1, v/v) and the appropriate amount of SPH in chloroform/methanol (2:1, v/v) were mixed and dried under a stream of O_2-free dry N_2 and the last traces were removed under high vacuum overnight. The samples were dispersed for 30 min at 55°C, temperature above the gel to liquid-crystalline phase transition of the mixture, in 1 ml for DSC and 15 ml for ^{31}P-NMR of 0.1 mM EDTA, 50 mM Mes, 100 mM NaCl, pH 6.0 with occasional mixing in a vortex mixer until obtaining a homogeneous and uniform suspension. Subsequently,

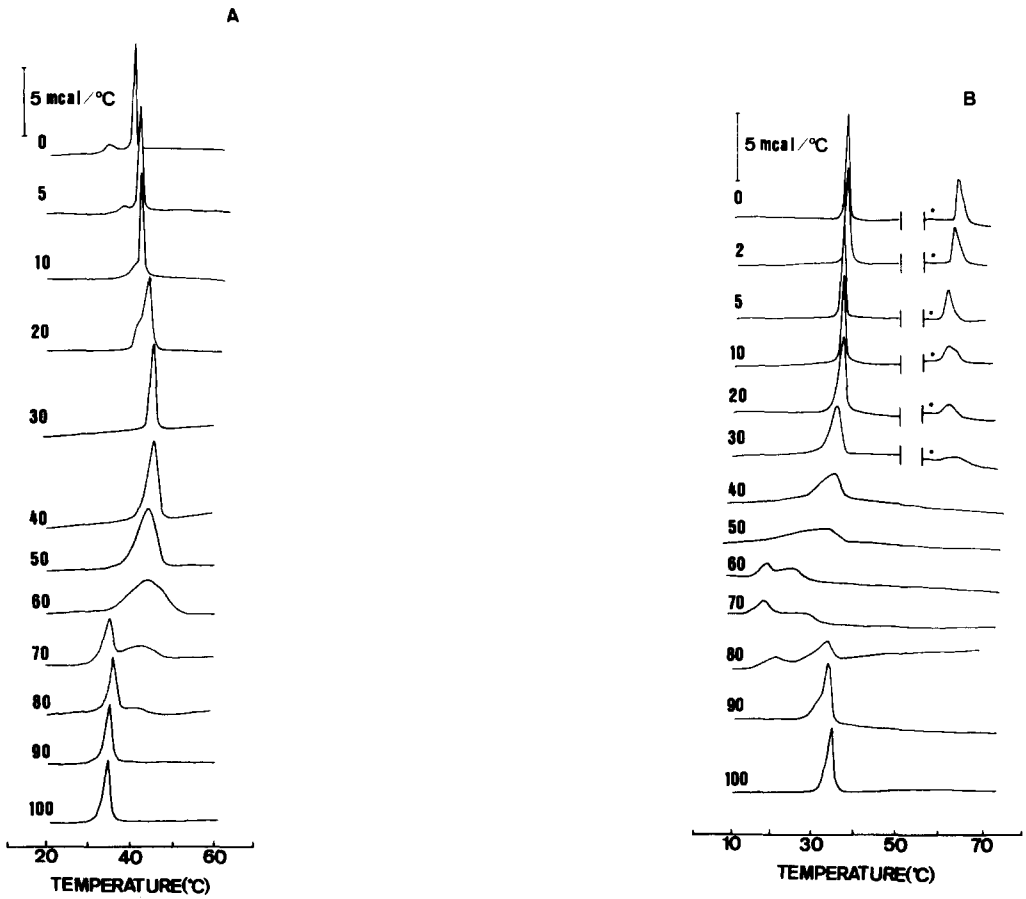

Fig. 1. Heating DSC calorimetric curves for systems containing (A) DPPC/SPH and (B) DEPE/SPH at pH 6.0. Molar percentages are indicated on the curves. In part B the sign * marks the parts of the thermograms corresponding to the liquid-crystalline to hexagonal H_{II} phase transition of the mixtures DEPE/SPH which were recorded at a sensitivity 4-fold greater than the thermograms corresponding to the gel to the liquid-crystalline phase transition.

the samples were centrifuged at 30 000 × g for 45 min in a Beckman LB-55M ultracentrifuge. The ratio of lipid to solvent was measured by weight before and after the preparation of the hydrated samples. In all cases water was found to be between 80 and 90% by weight, i.e, in a clear excess over what is needed for full hydration of the lipids.

2.2. Differential scanning calorimetry

Pellets for DSC were collected and placed into small aluminum pans, sealed and incubated for 48 h at 20°C before scanning in a Perkin-Elmer DSC-4 calorimeter using 10 μl of buffer as reference. The instrument was calibrated using indium as standard. The samples were scanned with a heating and cooling rate of 4°C/min at 1 mcal/s and the first scan was used for display. The samples were scanned between 10°C and 80°C.

After measurements, the pans were carefully opened and the samples were dissolved in chloroform/methanol 1:1 (v/v). The amount of phospholipid originally present was determined after subsequent perchloric acid hydrolysis as described before [23].

The incorporation of SPH into liposomes was measured by combining phosphorus assay [23] and the determination of the amine groups by the method of Benson and Hare [24]. The incorporation of SPH in the membranes was higher that 95% in all the samples used in this work.

2.3. ^{31}P-nuclear magnetic resonance

The samples for NMR were recorded in the Fourier transform mode on a Varian Unity 300 spectrometer (121 MHz for phosphorus) and the temperature was controlled to ±0.5 C°. All chemical shift values are quoted in parts per million (ppm) from pure lysophosphatidylcholine micelles (0 ppm), positive values referring to low-field shifts. All spectra were obtained in the presence of a gated-broad band decoupling (5 W input power during acquisition time) and accumulated free memory of 8 K data points, 1.3 s interpulse time and a 80° radio frequency pulse, were used. Prior to Fourier transformation an exponential multiplication was applied resulting in a 100 Hz line broadening. The residual chemical shift anisotropy, $\Delta\sigma$, was measured from the spectrum directly as the difference between the high- and low-field shoulders (the error of the measurement by this method was ± 3 ppm).

3. Results

3.1. Differential scanning calorimetry experiments

The effect of SPH, a molecule of biological importance, on the phase behavior of DPPC and DEPE, has been investigated by means of DSC and ^{31}P-NMR. We first describe the effect of SPH on the thermotropic phase transitions of the two phospholipids. Fig. 1A shows the effect of the incorporation of different amounts of SPH on the gel to liquid-crystalline phase transition of DPPC at pH 6.0, i.e., when SPH is fully protonated. We have selected this pH value to be sure about the full protonation of SPH, since the apparent pK of SPH is 9.1 in phospholipid mixtures [10], and pK_a values of 6.7 and 7.7 have been described in Triton micelles [25,26]. For the pure phospholipid, the gel to liquid-crystalline ($P_{\beta'} \rightarrow L_\alpha$) phase transition was detected at 41°C (T_c) and a pretransition ($L_\beta \rightarrow P_{\beta'}$) at 36°C, in good agreement with other reported values [27,28]. When SPH was added to DPPC the main T_c phase transition temperature of the phospholipid increased from 41°C in pure DPPC to approx. 45°C at a concentration of 30 mol% of SPH. At this SPH concentration a single sharp and narrow peak was found. This peak corresponds to an azeotropic mixture of DPPC and SPH, as it has been deduced from the phase diagram (see below). The temperature of the pretransition of DPPC was also affected by the presence of SPH as it can be observed in Fig. 1A. It increased as the amount of SPH increased, until disappearing at concentrations of SPH greater than 20 mol%. Concentrations higher than 30 mol% of SPH caused the broadening of the main transition peak and the decrease of its T_c, indicating non-ideal mixing, i.e., phase separation between DPPC and SPH. At concentrations of 70 mol% of SPH or higher, two different peaks were clearly discerned, one of them sharp at 31°C and the other one broad and weak, which progressively disappeared as the SPH concentration increased (Fig. 1A). Moreover, the sharp one, occurring at approx. 31°C, could be assigned to pure SPH, because it did not change as the SPH concentration increased showing a T_c identical to that of pure SPH.

The total enthalpy of the gel to liquid-crystalline transition of the different mixtures of DPPC and SPH is plotted in Fig. 2. The enthalpic values decreased progressively as SPH increased until approx. a concentration of 60 mol% of SPH in DPPC, approaching the value found for pure SPH. The concentration of 60 mol% of SPH in DPPC defines the boundary where one or two peaks are observed in the thermograms (see Fig. 1A). ΔH values of mixtures containing higher amounts of SPH, up to pure SPH, remained constant.

Aqueous dispersion of DEPE can undergo a gel to liquid-crystalline ($L_\beta \rightarrow L_\alpha$) phase transition at 37.1°C and in addition, a lamellar to hexagonal H_{II} ($L_\alpha \rightarrow H_{II}$) phase transition at 62.4°C (T_H) [29–32] as it is shown in the thermograms of pure DEPE (Fig. 1B, upper part). The transition enthalpy of the hexagonal H_{II} transition is much lower than the $L_\beta \rightarrow L_\alpha$ transition due to the fluid character of both the lamellar and

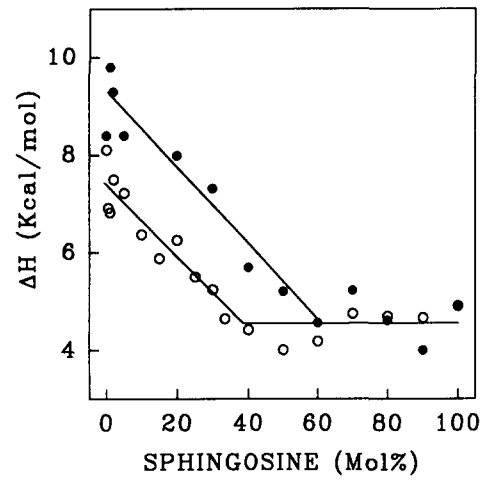

Fig. 2. Dependence of the enthalpy of the main gel to liquid-crystalline phase transition of mixtures of DPPC/SPH (•) and DEPE/SPH (○).

the hexagonal phases [33]. The effect of the incorporation of SPH in the DEPE phase transitions is shown in Fig. 1B. The phase transition temperatures decreased gradually as the concentration of SPH was increased, up to a concentration of 30 mol% of SPH, for which a T_c of 34.4°C was found. At the same time a broadening of the $L_\beta \rightarrow L_\alpha$ was observed, which is more evident at higher percentages of SPH. On the other hand, the $L_\alpha \rightarrow H_{II}$ phase was observed at SPH concentrations of 30 mol% and lower, being absent at greater concentrations, whereas T_H slightly decreased as the amount of SPH was increased. Between 50 mol% and 85 mol% of SPH broad transitions were observed, where it was possible to distinguish at least two different components. The transition temperatures of these two peaks were lower than the pure components, indicating the formation of non-ideal mixtures. At 90 mol% a peak and a shoulder were observed, at temperatures very close to that found for the transition of pure SPH.

The ΔH values of the different samples of DEPE and SPH are shown in Fig. 2. The behavior was similar to that of the mixtures of DPPC and SPH. It can be seen that ΔH decreased until a value of 4.4 kcal/mol at 40 mol% of SPH and remained constant until pure SPH (ΔH 4.9 kcal/mol). Again, and as it was found before for DPPC, at 40 mol% of SPH in DEPE the peak became broad where it was possible to distinguish at least two different components in the thermograms (Fig. 1B).

3.2. ^{31}P-nuclear magnetic resonance experiments

Dispersions of phospholipids can adopt several structures apart from the bilayer one, including micellar, hexagonal and cubic phases [34]. The ability of phospholipids to adopt these different structures is known as lipid polymorphism. These non-bilayer structures can greatly affect the functional behavior of the membrane [35]. Since lipid polymorphism has such a potential biological importance, we have studied the capacity of SPH to modulate the lipid polymorphism of both phosphatidylcholine (PC) and phosphatidylethanolamine (PE). In this way, the effect of SPH on the thermotropic phase behavior of DPPC and DEPE was also investigated by ^{31}P-NMR. Membrane phospholipids, when organized in bilayer structures, give rise to a characteristic asymmetrical ^{31}P-NMR line-shape with a high-field peak and a low-field shoulder presenting a residual chemical shift anisotropy ($\Delta\sigma$) of approx. 64 ppm in the gel state and approx. 45 ppm in the fluid state due to a increased ^{1}H-^{31}P dipolar interactions [36–38]. In the hexagonal H_{II} phase, due to rapid lateral diffusion of the phospholipids around the tubes of which this phase is composed, the chemical shift anisotropy is averaged resulting in a shape with reverse asymmetry when compared to the bilayer line-shape, i.e., a high-field shoulder and a low-field peak, and an approx. 2-fold reduction in the absolute value of $\Delta\sigma$ [29,39].

The origin of an isotropic peak in the ^{31}P-NMR spectra is not known with certainty since it may correspond to the formation of small vesicles, regions of the bilayer surface with a relative high curvature or it could be also originated by a separate phase, either cubic or an isotropic melt. Furthermore, ^{31}P-NMR results are to be considered with caution, since ^{31}P-NMR line-shape does not unambiguously sense phospholipid phase structure because changes in headgroup conformation can, at least theoretically, affect the spectral shape in a manner consistent with a phase change [40].

The ^{31}P-NMR spectra of pure DPPC showed an asymmetrical line-shape with a high-field peak and a low-field shoulder in the range of temperatures studied, indicating that DPPC was in a lamellar phase (Fig. 3A). At 30°C and 40°C it could be observed that the line-shape was broader ($\Delta\sigma$ was 63 ppm) than at higher temperatures, i.e., DPPC was in the gel state, whereas above 40°C DPPC was in the fluid state ($\Delta\sigma$ of 50 ppm), in accordance with the DSC results. The ^{31}P-NMR spectra of samples of DPPC containing 5 mol%, 10 mol% (not shown for briefness) and 33 mol% of SPH did not change significantly in the presence of SPH comparing with pure DPPC (Fig. 3B), indicating the presence of lamellar phases in the range of concentrations and temperatures studied. The $\Delta\sigma$ values increased at increasing SPH concentrations for the same temperature. There was an increase of approx. 12 ppm at 30°C and approx. 6 ppm at 55°C in $\Delta\sigma$, comparing the samples of pure DPPC and the sample containing 33 mol% of SPH. This data would indicate the possible existence of a reorientation and/or a change in mobility of the headgroup of the phospholipid in the presence of SPH.

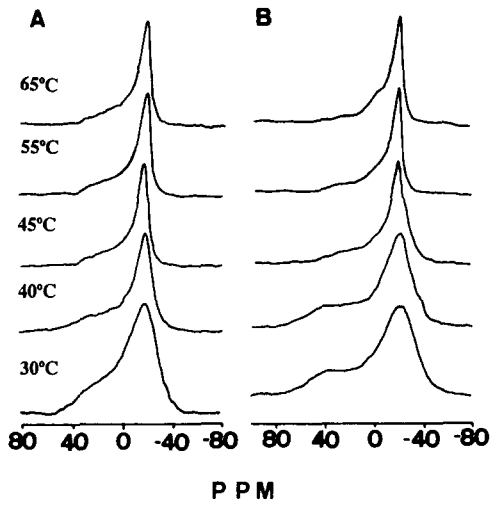

Fig. 3. ^{31}P-NMR spectra of (A) pure DPPC and containing (B) 33 mol% of SPH at the different temperatures as indicated. The spectra have been normalized in height.

The ^{31}P-NMR spectra of pure DEPE and containing different amounts of SPH are shown in Fig. 4. The ^{31}P-NMR of pure DEPE is shown in Fig. 4A. At 20°C and 35°C pure DEPE presented a characteristic asymmetrical line-shape with a high-field peak and a low-field shoulder corresponding to the gel phase ($\Delta\sigma$ of 64 ppm). At 45°C and 60°C the line-shape was narrower ($\Delta\sigma$ of 44 ppm) indicating that at this temperature DEPE is in a fluid state, in accordance with the DSC results shown above. However, at 63°C and 75°C DEPE presented an asymmetrical line-shape with a high-field shoulder and a low-field peak that indicated that, at these temperatures, DEPE was in the hexagonal phase H_{II} ($\Delta\sigma$ of 23 ppm). In the presence of 5 mol% of SPH and at 60°C, in contrast to what was found for pure DEPE, the line-shape of the ^{31}P-NMR spectrum corresponded mainly to a hexagonal H_{II} phase with a minor lamellar component (Fig. 4B), whereas at lower temperatures the mixture showed a characteristic lamellar phase. These data indicate that SPH induced a slight decrease of the $L_\alpha \rightarrow H_{II}$, in good agreement with the results reported by DSC. Between 5 mol% and 20 mol% of SPH (Figs. 4B, C and D, respectively), all the samples showed a similar behavior, i.e., hexagonal phases were observed at 60°C and lamellar phases at lower temperatures. However, at 20 mol%, and 50 mol% of SPH the spectra was characterized by a narrow and sharp peak centred at 0 ppm, i.e., the presence of isotropic phases (Figs. 4D and E). In the sample containing 50 mol% of SPH it was clear that a direct transition from a lamellar to an isotropic phase took place between 45°C and 60°C (Fig. 4E). As it was observed for the samples containing DPPC/SPH, mixtures of DEPE/SPH presented an increase in $\Delta\sigma$ of approx. 9 ppm at 20°C and at 45°C if we compare the samples containing pure DEPE and 50 mol% of SPH, indicating as before the possible existence of a reorientation and/or a change in mobility of the headgroup of the phospholipid in the presence of SPH.

3.3. Phase diagrams

Phase diagrams have been constructed on the basis of DSC and ^{31}P-NMR results. DSC onset temperatures for the gel to liquid-crystalline transition have been used as the primary data. Heating and cooling DSC scans were used to produce the solidus and fluidus lines, respectively, as described before [41].

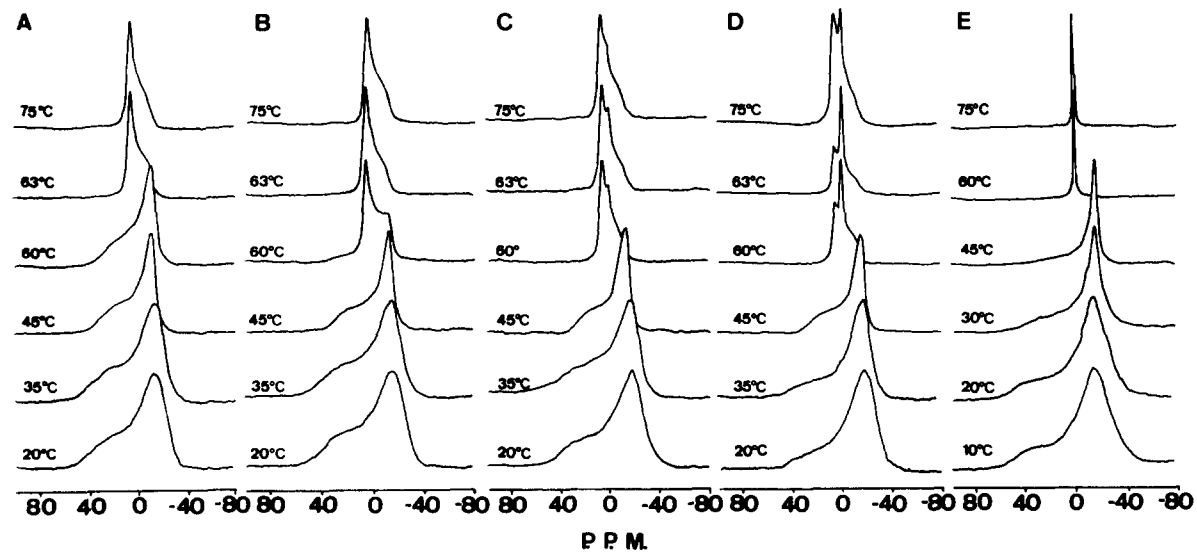

Fig. 4. ^{31}P-NMR spectra of (A) pure DEPE and containing (B) 5 mol% of SPH, (C) 10 mol% of SPH, (D) 20 mol% of SPH and (E) 50 mol% of SPH at the different temperatures as indicated. The spectra have been normalized in height.

The phase diagram corresponding to mixtures of DPPC with SPH is shown in Fig. 5A. It can be seen that increasing amounts of SPH in DPPC increased T_c reaching a maximum at approx. 44°C at a concentration of 30 mol% of SPH. This fact together with the shape of the peak observed, i.e., a single and sharp peaks, allow us to affirm that this composition corresponds to an azeotropic mixture. ^{31}P-NMR data shows that at SPH concentrations up to 33 mol% of SPH all these mixtures were lamellar. At higher concentrations of SPH a solid-solid phase separation occurred, beginning at 70 mol% of SPH with the presence of an eutectic point at approx. 90 mol% of SPH.

The phase diagram corresponding to mixtures of DEPE with SPH is shown in Fig. 5B. This phase diagram is different from that presented in Fig. 5A for the DPPC/SPH system. It can be observed here, that a good miscibility takes place up to approx. 10 mol% of SPH and no azeotropic point can be observed. At concentrations higher than 40 mol% of SPH a solid-solid phase separation took place being clear the formation of solid solutions, S1 and S2, and an eutectic point at 60 mol% of SPH.

4. Discussion

We have studied the interaction of a positively-charged single-chain molecule, SPH, with DPPC and DEPE by DSC and ^{31}P-NMR. SPH has only one hydrocarbon chain compared to the common phospholipid present in biomembranes which have two hydrocarbon chains.

From the phase diagram of DPPC/SPH it can be seen that the system shows an increase in T_c up to 30 mol% of SPH, where the phase transition becomes very cooperative (T_c occurs at several degrees above of the melting temperatures of the pure components). This peculiar behavior has been described as reflecting the formation of a maximum azeotropic point in the binary phase diagram of the mixture, which could appears as a consequence of strong and rather specific non-idealities in the lateral mixing of the two components [41,42]. It is remarkable that the elevation of the main transition temperature for mixtures of DPPC with SPH is not accompanied by a substantial increase in the transition enthalpy. Using the regular solution theory, which assumes that non-idealities in mixing are attributable entirely to enthalpic effects, we can calculate the transition enthalpies for the azeotropic mixture according to Eq. (1) [43]:

$$\Delta H^{e}_{total} = X_A^{max}\left(\Delta H^{o}_{A}\left[\left(T_{max}/T^{o}_{A}\right) - 1\right]\right) + \left(1 - X_A^{max}\right)\left(\Delta H^{o}_{B}\left[\left(T_{max}/T^{o}_{B}\right) - 1\right]\right) \quad (1)$$

where X_A^{max} is the molar fraction of the component A (DPPC, in our case) in the azeotropic mixture, and T_{max} the transition temperature of the azeotropic mixture. ΔH^{o}_{A}, ΔH^{o}_{B}, T^{o}_{A}, and T^{o}_{B}, are the enthalpies and the transition temperatures of the pure components. So that ΔH of the azeotropic mixture should exceed the ΔH of pure DPPC in approx. 120 cal/mol. Smaller increase of the transition enthalpy may be expected for these mixtures if the components also exhibit nonzero excess entropies of mixing.

A possible explanation for the behavior of this system is that, as previously described [44], the introduction of increasing amounts of cationic amphiphiles into PC bilayers may produce a reorientation of the PC headgroup, increasing progressively the average angle of the P-to-N axis with respect to the bilayer plane. This possibility is supported by the NMR results as commented above. This reorientation could decrease the effective cross-sectional area of the PC headgroup

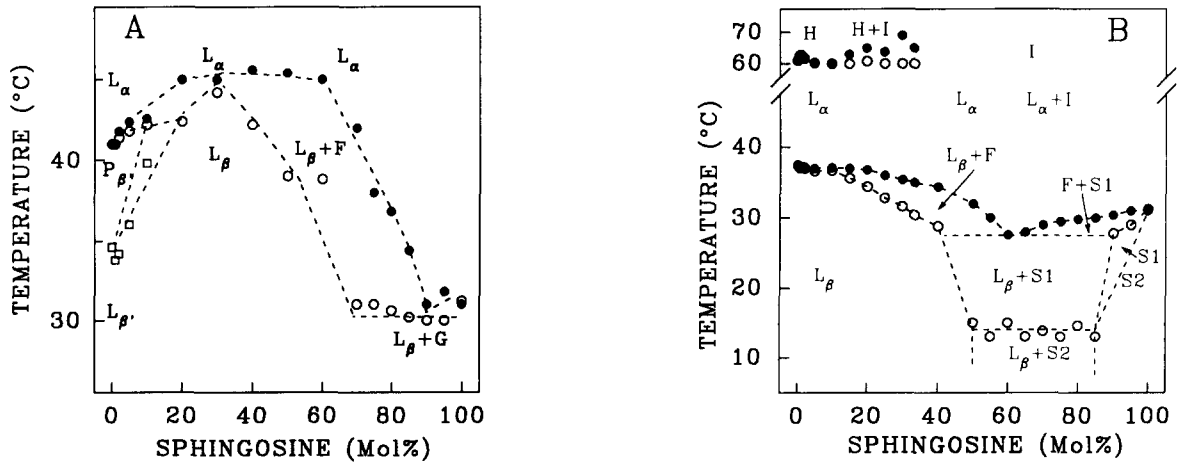

Fig. 5. Phase diagrams of the systems formed by (A) DPPC/SPH and (B) DEPE/SPH. Hollow and filled symbols were obtained from DSC heating and cooling scans, respectively, square symbols in part A denote the pretransition of DPPC and F and G denote fluid and gel phases, respectively.

by modifying steric and/or electrostatic interaction between headgroups [45,46]. In this way tighter intermolecular packing in mixed DPPC/SPH bilayer than in pure DPPC would be allowed, and therefore, a higher temperature of melting should be expected. Similarly, the transition temperature of DPPC could be also higher than that of pure SPH since the interaction with DPPC molecules will dilute its positive charges and therefore will diminish the repulsion effects.

The DEPE/SPH system shows, similarly to the DPPC/SPH system, a non-ideal mixing but it does not show an azeotropic point. It is possible then that SPH causes a smaller reorientation of PE headgroup than the PC headgroup, but it is more plausible that the DEPE headgroup is already small enough, even in its normal orientation, so that a shift in its orientation induced by cationic amphiphiles such as SPH would have comparatively small effects on intramolecular packing. This is further supported by the NMR results since the increase in $\Delta\sigma$ is lower in this samples than in the samples containing DPPC and SPH. This possibility is quite reasonable given that saturated PE occupy significantly smaller molecular areas in gel state bilayers than saturated PC [47–52].

On the other hand, the effect of high concentrations of monovalent ions on PE has been described to be an increase in the gel to fluid phase transition [53–55]. The electrostatic effect is not as important here, as it was found to be for mixtures of SPH with phosphatidylserine where an increase in the transition temperature was observed [10]. On the contrary, the presence of a compound with a lower T_c temperature induced a decrease in T_c of the mixture, probably by altering the hydrophobic interactions between the acyl chains of the phospholipid.

Low concentrations of SPH did not induce an appreciable shift of the lamellar to H_{II} hexagonal phase transition but high concentrations produced the appearance of isotropic phases as detected by ^{31}P-NMR (Fig. 4). At concentrations of SPH higher than 50 mol% a direct transition from L_β to an isotropic phase was observed. The mixture dimyristoylphosphatidylcholine/myristic acid (1:2, mol/mol) also may undergo a direct transition from gel lamellar to an isotropic phase, which has been characterized as a cubic phase [56]. This illustrates that even the H_{II} phase can be destabilized when the concentration of a molecule such as SPH is high enough. Nevertheless we cannot distinguish from our ^{31}P-NMR experiments whether micellar, cubic or other isotropic phases were present in these experiments.

The concentration of SPH at which effects on transition temperatures or bilayer stability are seen is higher than that found in natural membranes [12]. However, several of the studies to date on the effects of SPH on various enzymes use model vesicle or micelle systems as a vehicle for SPH [26] and it would be possible that the effects on the enzymes attributed to SPH could involve also alterations in lipid packing due to SPH. Apart from that, SPH concentration in vivo may be higher than expected in certain membrane domains, depending on membrane heterogeneity, SPH formation and lifetime, etc. The results presented here suggest that the incorporation of charged molecules into a membrane containing zwitterionic phospholipids can alter the thermotropic phase behavior of these lipids. These effects should be taken into account when considering the biological effects of cationic amphiphiles such as SPH.

Acknowledgements

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References

[1] Ohnishi, S.-I. and Ito, T. (1974) Biochemistry 13, 881–887.
[2] Luna, E.J. and McConnell, H.M. (1977) Biochim. Biophys. Acta 470, 303–316.
[3] Van Dijck, P.W.M., De Kruijff, B., Verkleij, A.J., Van Deenen, L.L.M. and De Gier, J. (1978) Biochim. Biophys. Acta 512, 84–96.
[4] Silvius, J.R. and Gagné, J. (1984) Biochemistry 23, 3232–3241.
[5] Grupe, R., Menzel, G., Steinberg, B., Zwanzig, M. and Göring, H. (1978) Stud. Biophys. 69, 161–173.
[6] Boggs, J.M., Rangaraj, G. and Koshy, K.M. (1986) Chem. Phys. Lipids 40, 23–34.
[7] Inoue, T., Miyakawa, K. and Shimozawa, R. (1986) Chem. Phys. Lipids 42, 261–270.
[8] Gallovà, J., Devínsky, F. and Balgavy, F. (1990) Chem. Phys. Lipids 53, 231–241.
[9] Inoue, T., Fukushima, K. and Shimozawa, R. (1990) Chem. Phys. Lipids 52, 157–161.
[10] López-García, F., Micol, V., Villalaín, J. and Gómez-Fernández, J.C. (1993) Biochim. Biophys. Acta 1153, 1–8.
[11] Hannun, Y.A. and Bell, R.M. (1989) Science 243, 500–507.
[12] Merrill, A.H. (1991) J. Bioenerg. Biomembr. 23, 83–104 (1991).
[13] Conkling, P.R., Patton, K.L., Hannun, Y.A., Greenberg, C.S. and Weinberg, J.B. (1989) J. Biol. Chem. 264, 18440–18444.
[14] Arnold, R.S. and Newton, A.C. (1991) Biochemistry 30, 7747–7754.
[15] Sakane, F., Yamade, K. and Kanoh, H. (1989) FEBS Lett. 255, 409–413.
[16] Lavie, Y. Piterman, O. and Liscovitch, M. (1990) FEBS Lett. 277, 7–10.
[17] Mullmann, T.J., Siegel, M.I., Egan, R.W. and Billah, M.M. (1991) J. Biol. Chem. 266, 2013–2016.
[18] Sohal, P.S. and Cornell, R.B. (1990) J. Biol. Chem. 265, 11746–11750.
[19] Lavie, Y. and Liscovitch, M. (1990) J. Biol. Chem. 265, 3868–3872.

[20] Kiss, Z. and Anderson, W.B. (1990) J. Biol. Chem. 265, 7345–7358.
[21] Hannun, Y.A., Loomis, C.R., Merrill, A.H. and Bell, R.M. (1986) J. Biol. Chem. 261, 12604–12609.
[22] Borek, C., Ong, A., Stevens, V.L., Wang, E. and Merrill, A.H. (1991) Proc. Natl. Acad. Sci. USA 88, 1953–1957.
[23] Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–471.
[24] Benson, J.R. and Hare, P.E. (1975) Proc. Natl. Acad. Sci. USA 72, 619–622.
[25] Bottega, R., Epand, R.M. and Ball, E.H. (1989) Biochem. Biophys. Res. Commun. 164, 102–107.
[26] Merrill, A.H., Nimkon, S., Menaldino, D., Hannun, Y.A., Loomis, C., Bell, R.M., Tyagi, S.R., Lambeth, J.D., Stevens, V.L., Hunter, R. and Liotta, D.C. (1989) Biochemistry 28, 3138–3145.
[27] Chapman, D., Williams, R. and Ladbrooke, B.D. (1967) Chem. Phys. Lipids 1, 445–461.
[28] Marsh, D. (1990) Handbook of Lipid Bilayers, CRC Press, Boca Raton, FL.
[29] Gallay, J. and De Kruijff, B. (1984) Eur. J. Biochem. 142, 105–112.
[30] Ghosh, R. and Seelig, J. (1982) Biochim. Biophys. Acta 691, 151–160.
[31] Cullis, P.R. and De Kruijff, B. (1978) Biochim. Biophys. Acta 513, 31–42.
[32] Tilcock, C.P.S. and Cullis, P.R. (1983) Biochim. Biophys. Acta 684, 212–218.
[33] Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420.
[34] Cullis, P.R., Hope, M.J., De Kruijff, B, Verkleij, A.J. and Tilcock, C.P.S. (1985) in Phospholipid and Cellular Regulation (Kuo, J.F., ed.), Vol. 1, pp. 1–60, CRC Press, Boca Raton, FL.
[35] Van Echteld, C.J.A., Van Sitgt, R., De Kruijff, B., Leunissen-Bijvelt, J., Verkleij, A.J. and De Gier, J. (1981) Biochim. Biophys. Acta 648, 287–291.
[36] Killian, J.A. and De Kruijff, B. (1985) Biochemistry 24, 7881–7890.
[37] Seelig, J. and Gally, H.V. (1976) Biochemistry 15, 5199–5204.
[38] De Kruijff, B., Cullis, P.R., Verkleij, A.J., Hope, M.J., Van Echteld, C.J.A., Taraschi, T.F. (1985) in The Enzyme of Biological Membranes (2nd Edn.) (Martonosi, A.D., ed.), Vol. 1, pp. 131–204, Plenum Press, New York.
[39] Seelig, J. (1987) Biochim. Biophys. Acta 515, 105–140.
[40] Tayer, A.M. and Kohler, S.J. (1980) Biochemistry 20, 6831–6834.
[41] Mabrey, S. and Sturtevant, J. (1977) Biochim. Biophys. Acta 486, 444–450.
[42] Koynova, R.D., Tenchov, B.G., Quinn, P.J. and Laggner, P. (1988) Chem. Phys. Lipids 48, 205–214.
[43] Silvius, J.R. (1991) Biochim. Biophys. Acta 1070, 51–59.
[44] Scherer, P.G. and Seelig, J. (1989) Biochemistry 28, 7720–7728.
[45] Boggs, J.M. (1987) Biochim. Biophys. Acta 906, 353–404.
[46] Dill, K.A. and Stiger, D. (1988) Biochemistry 27, 3446–3453.
[47] Inoko, Y. and Mitsui, T. (1978) J. Phys. Soc. Jap. 44, 1918–1924.
[48] Janiak, M.J., Small, D.M. and Shipley, G. (9179) J. Biol. Chem. 254, 6068–6078.
[49] Hauser, H., Pascher, I., Pearson, R.H. and Sundell, S. (1981) Biochim. Biophys. Acta 650, 21–51.
[50] Lis, L.J., McAlister, M., Fuller, N., Rand, R.P. and Parsegian, V.A. (1982) Biophys. J. 37, 657–666.
[51] Seddon, J.M., Cevc, G., Kaye, R.D. and Marsh, D. (1984) Biochemistry 23, 2634–2644.
[52] Cevc, G., Seddon, J.M. and Marsh, D. (1987) Far. Disc. Chem. Soc. 81. 179–190.
[53] Harlos, K. and Eibl, H. (1981) Biochemistry 20, 2888–2892.
[54] Seddon, J.M., Cevc, G. and Marsh, D. (1983) Biochemistry 22, 1280–1289.
[55] Epand, R.M. and Bryszewska, M. (1988) Biochemistry 27, 8776–8779.
[56] Heimburg, T., Ryba, N.J.P., Würz, U. and Marsh, D. (1990) Biochim. Biophys. Acta 1025, 77–81.