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Spin label EPR study of the effects of monovalent cations, anions, and chaotropics on DPPC multilayers

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Abstract

The electron paramagnetic resonance (EPR) spectroscopy with the spin-labeling technique is used to investigate the effects of monovalent ions on multibilayer dispersions of dipalmitoylphosphatidylcholine (DPPC). Cations of chloride salt (Li^+, Na^+, K^+ and Cs^+) and anions of potassium salt (Br^-, Cl^- and NO_3^-) at the concentration of 1 M do not affect both the molecular order and the packing of the phospholipid acyl chains in the different phases compared to DPPC dispersions in buffer. Moreover, they leave unaffected the characteristics of the main transition, whereas the pre-transition temperature increases of about 2°C in the presence of cations and changes in the order $NO_3^- < Br^- <$ buffer $< Cl^-$ in the presence of anions. The anions that exhibit pronounced chaotropic properties (I^-, SCN^-) result the most effective in perturbing the bilayer. In fact, DPPC dispersions in 1 M of these salt solutions do not show the pre-transition and have the main one shifted to lower temperature in the order: $SCN^- < I^-$ < buffer. Furthermore, the spin-label EPR results on the lipid chain dynamics indicate the presence of a flexibility gradient both in DPPC/buffer and in DPPC/chaotropic systems. However, the chaotropic anions influence the DPPC hydrocarbon chains in the gel phase in a manner such that interpenetration or interdigitation of the terminal methyl groups from opposing monolayers is likely to occur.

Keywords: DPPC; Chaotropic anion; Spin label; EPR

1. Introduction

Many structural and functional properties of biological membranes can be influenced, in principle, by the interaction of membrane components with ions present in the dispersion medium of the cell. In this respect, extensive investigations have been carried out on the interaction between monovalent ions in aqueous solutions and bilayer model membranes made of neutral phospholipids [1–20]. From these studies it comes out that the effects of monovalent ions are small but different for cations and anions. In particular, the monovalent chaotropic anions, i.e., agents able to break up the water structure near the polar headgroups [21,22], seem to have significant capability in perturbing the zwitterionic lipid bilayers [4–6,11,13–17,19,20]. However, some experimental studies have pointed out that the chaotropic anions influence the headgroup structure of phosphatidylcholines to some extent without disrupting the long range organization of the lipid bilayer configuration [16,19,20]. Other authors, instead, report that molar solution of the chaotropic anion SCN^- also influences the bilayer structure and the packing of the phases of PCs [13,14,17].

In order to get more insight into the monovalent ions–neutral lipid bilayer interaction, we have investigated the effect of various monovalent ions on the lamellar phases of DPPC multilayers. Indeed, chloride salts with alkali metal cations: Li^+, Na^+, K^+, Cs^+; anions of potassium salts: Br^-, Cl^-, NO_3^-, and chaotropic anions of potassium salts: I^-, SCN^- have been used. Due to the slight effects produced by ions, the concentration of 1 M, which is the

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PCs, phosphatidyl-cholines; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; CBS, 10 mM citrate buffer solution at pH 5; BBS, 10 mM borate buffer solution at pH 9; KSCN, potassium thiocyanate; EPR, electron paramagnetic resonance; ^2H- and ^{31}P-NMR, deuterium and phosphorus-31 nuclear magnetic resonance; DSC, differential scanning calorimetry; *n*-NSA, *n*-nitroxide stearic acid spin-labeled molecule; DTBN, di-*tert*-butyl-nitroxide spin probe.

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most effective in influencing the properties of DPPC bilayers [2,4,5,7,9,12–14,16,17], has been used. The spin-label EPR spectroscopy is employed with the aim to compare both the thermotropic phase behaviour and the lipid chain order and fluidity of DPPC multilayers in aqueous buffer and in the presence of different ions. For this purpose we used the nitroxide stearic acid spin labeled at different positions along the acyl chain, *n*-NSA, and the spin probe DTBN, that partitions between the aqueous and the fluid hydrocarbon phases of phospholipid bilayers [23–25]. We find that all the cations and the anions Br^-, Cl^- and NO_3^- have negligible effect on the molecular order and on the packing density of the DPPC acyl chains. Moreover, they leave unaffected the characteristics of the main transition and shift in opposite direction the temperature of the pre-transition of DPPC. On the other hand, the chaotropic anions at 1 M suppress the pre-transition and lower the temperature of DPPC main transition in an order that correspond to their ability to break-up the structure of water, i.e., $SCN^- < I^- <$ buffer. Moreover, our spin-label EPR measurements on the lipid chain order, indicate the presence of a fluidity gradient both in DPPC/buffer and in DPPC/chaotropic systems. However, in the presence of chaotropics a slight interdigitation of the hydrocarbon tails around the bilayer midplane is more plausible rather than a fully interdigitated gel phase as already reported [13,14,17].

These results are discussed in terms of the different influence of monovalent cations, anions, and chaotropics on the structure of the solvent in the double layer regions and on the mechanism of binding to the DPPC headgroup moieties.

2. Materials and methods

2.1. Chemicals

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased from Sigma (St. Louis, MO, USA) and used without further purification. The stearic acid spin labels 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy (5-NSA), 2-(5-carboxypentyl)-4,4-dimethyl-2-undecyl-3-oxazolidinyloxy (7-NSA), 2-(8-carboxyoctyl)-4,4-dimethyl-2-octyl-3-oxazolidinyloxy (10-NSA), 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy (12-NSA), 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy (16-NSA) and the spin probe di-*tert*-butyl nitroxide (DTBN) were obtained from Aldrich (Milwaukee, WI, USA).

The citric acid and the trisodium citrate 5,5-hydrate for the 10 mM citrate buffer solutions (CBS) at pH 5, the boric acid and the NaOH for the 10 mM borate buffer solutions (BBS) at pH 9, and the monovalent ions were from Merck (Darmstadt, Germany) except for potassium thiocyanate (KSCN) obtained from Aldrich. Distilled water was used throughout.

2.2. Preparation of aqueous lipid dispersions

Multilayers of DPPC spin labeled with *n*-NSA were prepared by first mixing the lipids with 1% by weight of spin labels in chloroform. The solvent was evaporated under N_2(g) and the resultant lipid film was kept under vacuum overnight to remove any remaining traces of $CHCl_3$. Citrate or borate buffered solutions at 1 M salt concentration of monovalent ions at pH 5 or 9 were added to hydrate the film to a final lipid concentration of 10% wt. The lipids were then dispersed by heating and vortexing at 45°C, i.e., above the main lipid phase transition temperature and the suspensions were finally transferred to a 1 mm (i.d.), 100 μl glass capillary tubes and flame sealed. The pH-values of 5 and 9, far removed from the pK of the fatty acids, were chosen to ensure that the position of the nitroxide reporter group of the labels in the bilayer be precisely located. Indeed, at pH 5 the carboxylic groups of the fatty acid spin labels are fully protonated so that they are attached near the negative charges of the PO^- groups present on DPPC polar heads. In this situation the nitroxide would probe the glycerol backbone region. At pH 9, instead, the interaction between the fully dissociated $-COO^-$ groups and the positive charge on the choline quaternary ammonium group forces the nitroxide in the polar zone of DPPC bilayer ([18], Fig. 5 in Ref. [26]).

Dispersions of DPPC spin labeled with DTBN were prepared as described above except that the hydration of the lipid film was done with a spin-labeled salt solutions and the label/lipid molar ratio was of 1:400.

2.3. EPR measurements

EPR spectra were acquired using a 9 GHz Bruker spectrometer model ER 200D-SRC and digitized with the spectrometer's built-in microcomputer using OS-9-compatible ESP 1600 spectral acquisition software. Sample capillaries were inserted in a standard 4 mm diameter EPR quartz tube containing light silicon oil for thermal stability and centered in a TE_{102} rectangular EPR cavity (ER 4201, Bruker). Measurements on samples incubated at 10°C for 24 h were performed at thermal equilibrium starting from low temperatures. The sample temperature was controlled to within 0.5°C with a variable temperature controller (model ER 4111VT, Bruker). Conventional, in phase, absorption EPR spectra were recorded well below saturation at a power of 10 mW using a 100 kHz field modulation frequency for phase sensitive detection. 1 $G_{p\text{-}p}$ and 0.25 $G_{p\text{-}p}$ as amplitude of the magnetic field modulation signal were used for the *n*-NSA and for the DTBN spin-labeled samples, respectively.

2.4. Spectroscopic data analysis

A suitable spectral parameter that characterizes the EPR spectrum of randomly oriented nitroxide stearic acid spin

labels, *n*-NSA, is the outer hyperfine splitting, $2T'_{\parallel}$. It gives a relative measure of the local order of the lipid bilayer: large $2T'_{\parallel}$ values correspond to an ordered spin-label environment while small values correspond to a more disordered one. It reflects segmental lipids dynamics, too (chapters 12 and 13 in Refs. [23,24]).

When the small hydrophobic spin probe DTBN is dissolved in a lipid dispersion, it partitions between the aqueous and fluid hydrocarbon phases [23–25]. The corresponding EPR spectrum is the superposition of two isotropic triplets arising from DTBN in the two environments. As membranes have greater viscosity and smaller polarity than water, there are small differences in the isotropic hyperfine coupling constant, T_o, and in the isotropic *g*-factor, g_o, of the EPR spectra. This leads to a partial spectral resolution so that the resonance line at high magnetic field ($m_I = -1$) is clearly resolved at 9 GHz (inset Fig. 1B). Moving toward high fields, first the label signal in the lipid region (H_L) is seen and then the signal in the aqueous phase (H_W). An estimation of the fraction of DTBN in the fluid hydrophobic region of the bilayer can be obtained by evaluating the partition coefficient, $P_C = H_L/(H_L + H_W)$. Note that the corrections of the contributions of both the ^{13}C satellite resonances and the line width have been neglected in calculating P_C. The partition coefficient gives a relative measure of membrane fluidity [23–25]. Plots of $2T'_{\parallel}$ and P_C vs. temperature are used to detect bilayer phase transitions [23–25,27].

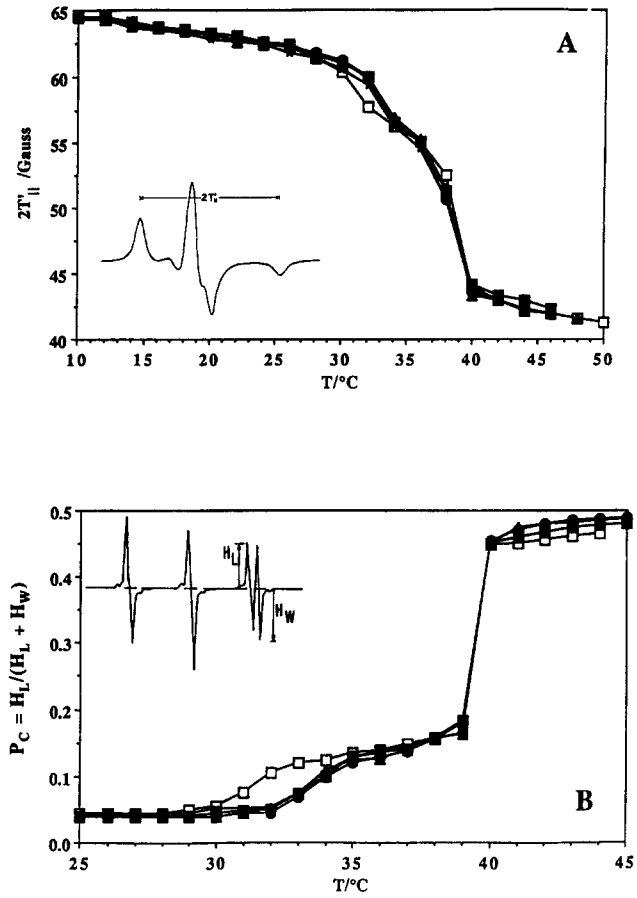

Fig. 1. (A) Temperature dependence of the outer hyperfine splitting, $2T'_{\parallel}$, of the spin label 5-NSA in multibilayer dispersions of DPPC in 10 mM citrate buffer solution at pH 5 (□) and in presence of LiCl (■), NaCl (●), KCl (▲) and CsCl (*) at the concentration of 1 M. Inset: ESR spectrum of the spin label 5-NSA in DPPC multilayers dispersed in 10 mM citrate buffer solution at pH 5 in the gel phase at 10°C. Total scan range = 100 G. (B) Temperature dependence of the partition coefficient, $P_C = H_L/(H_L + H_W)$, of the spin probe DTBN in multilayers of DPPC dispersed as in (A). Inset: ESR spectrum of the spin probe DTBN in DPPC multilayers dispersed in 10 mM citrate buffer solution at pH 5 at 45°C, i.e., above the main phase transition. Total scan range = 100 G.

3. Results

3.1. Monovalent cations

The plots of the outer hyperfine splitting, $2T'_{\parallel}$, vs. temperature for the spin label 5-NSA in DPPC multilamellar dispersions both in CBS and in 1 M solution of LiCl, NaCl, KCl and CsCl are reported in Fig. 1A. It is interesting to note that the label in the lipid bilayers undergoes the same large variation of the degree of its anisotropic motion at all the electrolytes considered. In fact, $2T'_{\parallel}$ decreases from about 65 to 42 Gauss as the temperature is raised from 10 to 50°C, respectively. This means that the monovalent cations in solution does not influence the order of the microenvironment tested by 5-NSA at pH 5.0, i.e., the region between the glycerol backbone and the beginning of the fatty acid acyl chains of DPPC.

Moreover, in CBS the spin label 5-NSA reveals the pre-transition, $L_{\beta'} \rightarrow P_{\beta'}$, of DPPC as a step drop of $2T'_{\parallel}$ vs. temperature around $T_p = 31$°C and the main transition, $P_{\beta'} \rightarrow L_{\alpha}$, as another drop at about $T_m = 39$°C (Fig. 1A and Table 1). The characteristics of these phase transitions, i.e., temperature, amplitude and width, are in agreement with the experimental method and the mesophase used [27,28].

The presence of 1 M solution of chloride salt of alkali metal cations shifts upward to 33°C the temperature of the pre-transition, while it has no effect on the major transition (Fig. 1A and Table 1).

Almost the same results are obtained from the temperature dependence of the partition coefficient, P_C, of DTBN in DPPC dispersions both in absence and in presence of 1:1 electrolyte reported in Fig. 1B. For any dispersion medium considered, the values of P_C range from 0.05 to 0.5 as the temperature is varied from 25 to 45°C. In other words, the permeability characteristics of DTBN in multilayers of DPPC are not affected by the monovalent cations in the dispersion medium.

As concerns the thermotropic phase behaviour of the DPPC/buffer system, the spin probe DTBN reveals the pre-transition at about 32°C and the main one at 39.5°C.

Table 1
Temperatures of the pre-, T_p, and main, T_m, phase transitions for DPPC multibilayers dispersed in 10 mM citrate buffer solution at pH 5 or 1 M of monovalent ions as detected with the spin labels 5-NSA and DTBN

Salt solution	5-NSA		DTBN	
	T_p (°C) [a]	T_m (°C) [a]	T_p (°C) [a]	T_m (°C) [a]
Buffer	31.0	39.0	32.0	39.5
LiCl	33.0	39.0	33.5	39.5
NaCl	33.0	39.0	33.5	39.5
KCl	33.0	39.0	33.5	39.5
CsCl	33.0	39.0	33.5	39.5
KBr	30.0	39.0	30.5	39.5
KNO_3	29.0	39.0	29.5	39.5
KI	–	37.5	–	38.5
KSCN	–	37.0	–	37.5

[a] The error on both T_p and T_m is ±0.5°C.

From Fig. 1B can be seen, once again, that the presence of salts does not influence the characteristics of the main transition, whereas T_p is increased of about 2°C, no matter of the cation present in the dispersion medium (Table 1). Note that the discrepancy in the temperature values of either T_p or T_m, revealed by the two spin labels 5-NSA and DTBN, is due to the different probe methods [28].

3.2. Monovalent anions

In Figs. 2A and 2B is given the temperature dependence of $2T'_{\parallel}$ and of P_C for the spin labels 5-NSA and DTBN, respectively, in DPPC multilamellar dispersions in CBS and in 1 M solutions of KBr, KCl and KNO_3. Both parameters undergo the same variations with the temperature for all the anions considered. In fact, $2T'_{\parallel}$ varies from about 65 to 42 Gauss in the temperature range 10–50°C and P_C goes from 0.05 to 0.5 as the temperature is raised from 25 to 45°C, respectively. It is interesting to note that the ranges of variation of $2T'_{\parallel}$ and P_C are the same of those reported for the cations (compare Figs. 1 and 2). Thus, neither the order nor the fluidity of DPPC multilayers are affected by the presence of monovalent cations and anions in the dispersion medium.

The trend of $2T'_{\parallel}$ vs. temperature in Fig. 2A shows that for DPPC/anion systems the characteristics of the main transition remain unchanged, whereas in presence of NO_3^- and Br^- the pre-transition is shifted to lower temperatures compared to those in CBS and in solution with KCl, being 29 and 30°C for DPPC/NO_3^- and DPPC/Br^-, respectively (Table 1).

The same conclusions hold for the phase transition behaviour detected by DTBN. In fact, the plots of P_C as a function of temperature in Fig. 2B reveal that the main transition is not influenced for the DPPC/anion systems, while the temperatures of the pre-transition are 29.5 and 30.5°C for DPPC/NO_3^- and DPPC/Br^-, respectively (Table 1).

3.3. Monovalent chaotropic anions

The temperature dependence of $2T'_{\parallel}$ of the spin label 5 – NSA in DPPC dispersions in buffer and in media containing 1 M of monovalent chaotropic anions I^- and SCN^- is given in Fig. 3A. The most striking feature of these plots is the difference between the behaviour of DPPC multilayers dispersed in CBS and that in the salt solutions. Firstly, there is the reduced order detected from the spin label in DPPC/salt systems for temperatures below the main transition. In fact, $2T'_{\parallel}$ is reduced from about 65 to 62 Gauss at 10°C and it remains systematically lower up to the main transition.

A second difference is in the thermotropic phase properties. The trend of $2T'_{\parallel}$ vs. temperature in presence of chaotropic anions shows both the lack of any pre-transitional behaviour and a shift to lower temperatures of the main transition that become 37.5 and 37.0°C for DPPC/KI and DPPC/KSCN, respectively (Fig. 3A and Table 1).

The plots of P_C vs. temperature reported in Fig. 3B

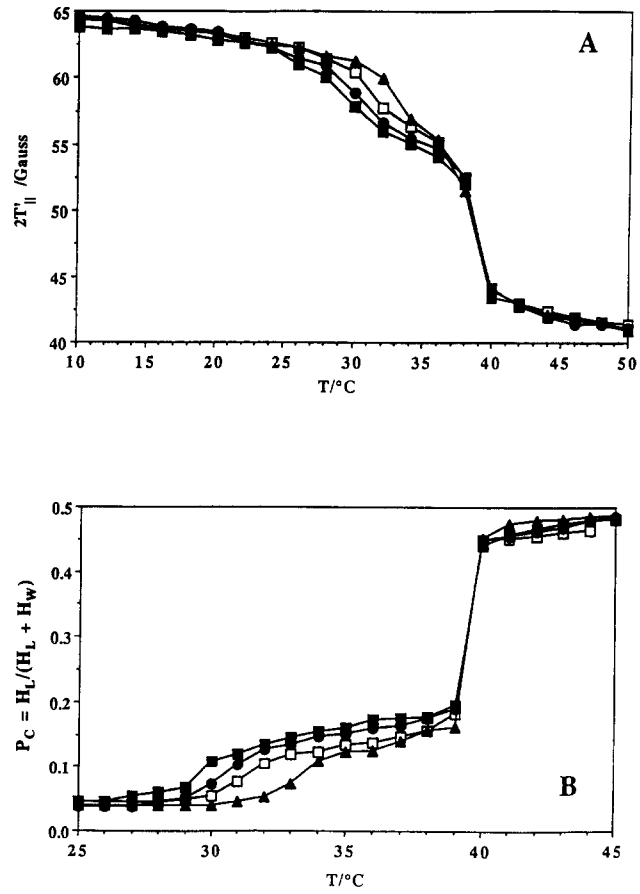

Fig. 2. (A) Temperature dependence of the outer hyperfine splitting, $2T'_{\parallel}$, of the spin label 5-NSA in multibilayer dispersions of DPPC in 10 mM citrate buffer solution at pH 5 (□) and in presence of KBr (●), KCl (▲) and KNO_3 (■) at the concentration of 1 M. (B) Temperature dependence of the partition coefficient, $P_C = H_L/(H_L + H_W)$, of the spin probe DTBN in multilayers of DPPC dispersed as in (A).

show an enhancement of the partition of DTBN in DPPC/chaotropic systems compared to that in CBS, particularly in the gel phase. As can be seen, at $T = 10°C$ the values of P_C change from 0.05 for DPPC/buffer system to ≈ 0.15 for DPPC/chaotropic systems. In the presence of chaotropics the P_C-values remain higher compared to those in buffer for temperatures in the gel phase.

As concerns the thermal properties of DPPC multilayers dispersed in molar solution of chaotropic anions, from Fig. 3B it is again evident that the pre-transition is not observed and the main one is shifted to lower temperatures at 38.5 and 37.5°C for KI and KSCN, respectively (Table 1).

3.4. Monovalent ions and lipid chain mobility

In order to see whether or not the phospholipid chains of DPPC multilayers are influenced by the presence of different salts in the dispersion medium, we have used stearic acids spin labeled at different positions down the acyl chain (*n*-NSA, $n = 5$, 7, 10, 12, and 16). The spectral anisotropy revealed by the spin labels throughout the entire length of the chain of DPPC multilayers dispersed in media containing 1 M of univalent either cations Li^+, Na^+, K^+ and Cs^+ or anions Br^-, Cl^- and NO_3^- is identical to that in DPPC/buffer system in the whole temperature range investigated. This means that the segmental chain mobility of DPPC is not affected by the presence of either cations or anions. On the other hand, as indicated by the results obtained with 5-NSA and DTBN, the chaotropic anions affect the chain packing of DPPC in the gel phase.

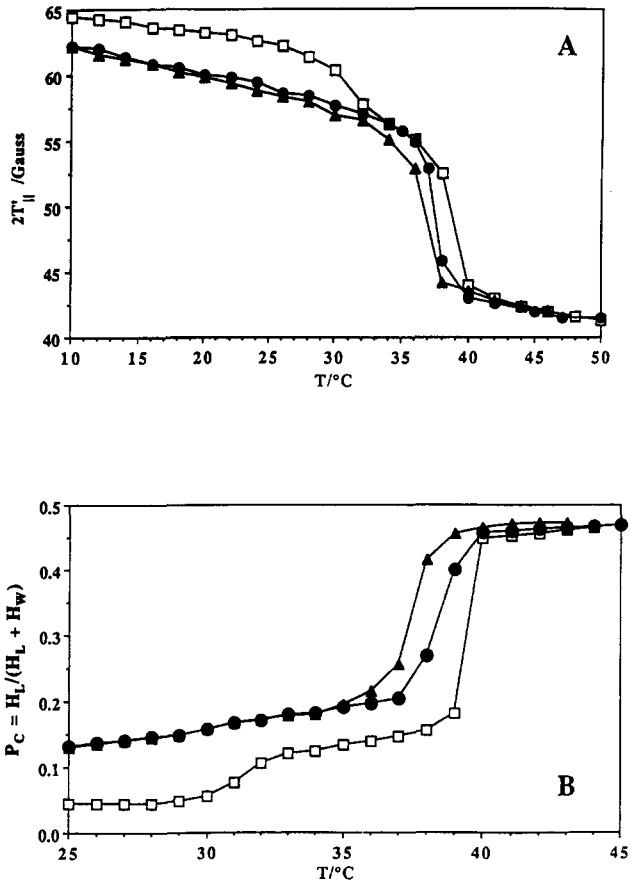

Fig. 3. (A) Temperature dependence of the outer hyperfine splitting, $2T'_{\parallel}$, of the spin label 5-NSA in multibilayer dispersions of DPPC in 10 mM citrate buffer solution at pH 5 (□) and in presence of KI (●) and KSCN (▲) at the concentration of 1 M. (B) Temperature dependence of the partition coefficient, $P_C = H_L/(H_L + H_W)$, of the spin probe DTBN in multilayers of DPPC dispersed as in (A).

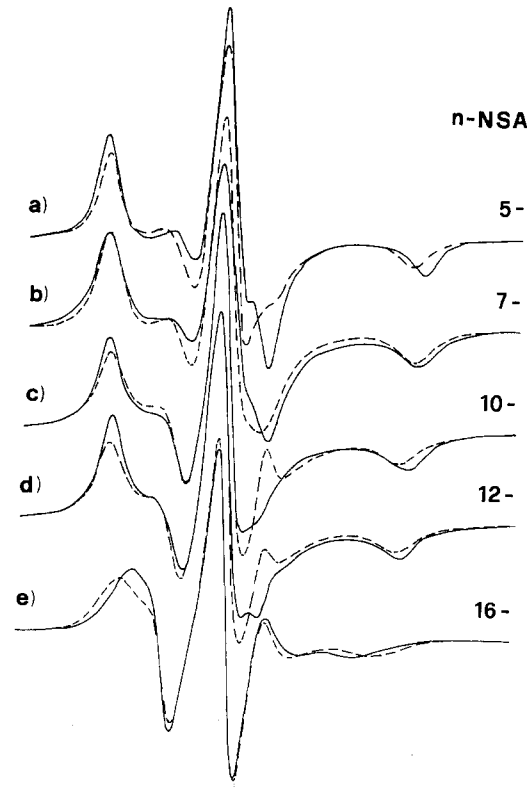

Fig. 4. ESR spectra of different positional isomers of the nitroxide stearic acid spin label (a) 5-NSA, (b) 7-NSA, (c) 10-NSA, (d) 12-NSA, and (e) 16-NSA, in multibilayer dispersions of DPPC in 10 mM citrate buffer solution at pH 5 (full line) and in 1 M solution of KSCN (dashed line). Spectra recorded in the gel phase at 10°C. Total scan range = 100 G.

The EPR spectra of *n*-NSA recorded in the gel phase at 10°C for DPPC multilayers in CBS and in presence of 1 M KSCN are reported in Fig. 4. By comparing the spectra, it is evident that the spin labels in both systems undergo some motion in the slow motion regime of the EPR time-scale leading the outermost lines to move in and the linewidths to become broader as the doxyl group is moved toward the hydrocarbon end (spectra from (a) to (e) in Fig. 4). However, it is interesting to note that, for a given label position with $n = 5$, 7, 10, 12, the spectral anisotropy is slightly greater for DPPC multilayers in aqueous buffer than for those in KSCN and this situation is reversed with 16-NSA. This behaviour is maintained for temperatures throughout the gel phase. In fact, as the temperature is raised, the spectral anisotropy decreases continuously on

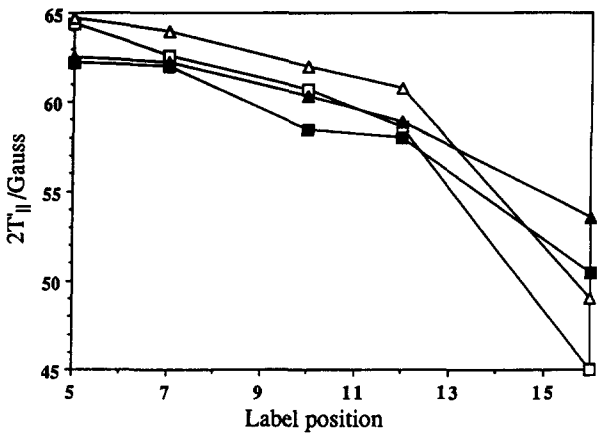

Fig. 5. Outer hyperfine splitting, $2T'_{\parallel}$, as a function of the label position, n, along the chain of the nitroxide stearic acid spin labels (n-NSA) in multilayer dispersions of DPPC in 10 mM citrate buffer solution at pH 5 (squares) and in 10 mM borate buffer solution at pH 9 (triangles). Open symbols correspond to buffers and filled symbols to 1 M KSCN. $T = 10°C$.

proceeding toward the terminal methyl end of the chain for both DPPC/buffer and DPPC/KSCN systems, although it is always lower in presence of KSCN and a slight increase of the degree of the molecular order of 16-NSA in DPPC/KSCN system compared to that in DPPC/buffer system is still evident. The other chaotropic salt investigated, namely KI at 1 M, influences the lipid chain motion as KSCN does.

Qualitatively, the results obtained with n-NSA indicate that a gradient of increased motional freedom along the hydrocarbon chains exists in both DPPC/CBS and DPPC/chaotropic systems in the gel phase.

Quantitatively, the flexibility profiles for DPPC multilayers dispersed in buffered solutions at pH 5 or 9 with and without 1 M KSCN are compared by means of $2T'_{\parallel}$ in the EPR spectra of the spin labels n-NSA in Fig. 5. For DPPC dispersions in CBS at 10°C, the values of $2T'_{\parallel}$ decrease systematically on proceeding down the chain, being ≈ 65, 60.7 and 45 Gauss at the C-5, C-10 and C-16 positions, respectively. For DPPC dispersions at pH 5 with 1 M KSCN the values of $2T'_{\parallel}$ are somewhat smaller and decrease steadily with the position of the spin label down the chain. In fact, the values of $2T'_{\parallel}$ are 62.2 and 62 Gauss at the C-5 and C-7 positions, decrease to 58.4 and 57.9 Gauss from the C-10 to the C-12 positions and finally to 50.5 Gauss at the C-16 position. At pH 9 we obtained almost the same results as at pH 5, albeit the values of $2T'_{\parallel}$ were a little higher than those in CBS as the nitroxide is located upper in the bilayer. Indeed, for the DPPC/BBS systems the values of $2T'_{\parallel}$ are 64.7, 63.9, 62, 60.8 and 49 Gauss for 5-, 7-, 10-, 12-, and 16-NSA, respectively, whereas, the corresponding values for the DPPC/1 M KSCN systems in buffered solutions at pH 9 are 62.5, 62.2, 60.3, 59, and 53.5 Gauss. Therefore, Fig. 5 shows that $2T'_{\parallel}(\text{KSCN}) < 2T'_{\parallel}(\text{buffer})$ for $5 < n < 12$ and $2T'_{\parallel}(\text{KSCN}) > 2T'_{\parallel}(\text{buffer})$ with 16-NSA. Moreover, the use of methyl ester n-NSA does not change the results, too. The measurements at different pH-values and with methyl ester n-NSA have been performed in order to rule out the possibility that a change in location of the probe in the bilayer was responsible for the increase in order observed with 16-NSA in DPPC/chaotropic systems.

In the fluid phase, i.e., for temperatures above the main transition, the spectra of the spin labels n-NSA (not shown) in DPPC/buffer and in DPPC/chaotropic systems are principally in the fast motion regime and posses axial anisotropy, which is characteristic of a liquid-crystalline phase. Further, any difference in the spectral anisotropy was absent so that the segmental mobility was comparable throughout the chain length in both environments.

4. Discussion

4.1. Monovalent ions and thermotropic phase behaviour of DPPC bilayers

As expected for the neutral lipids–monovalent ions interaction, our spin-label EPR results confirm that, in general, 1 M of monovalent ions have little effects on the thermal properties of DPPC bilayers [4,5,12,13,18]. Nevertheless, the DPPC thermotropic phase behaviour is influenced by the type of salt present in the dispersion medium.

Below we argue that the observed differences in the thermal phase behaviour are due to the different effect of monovalent cations, anions and chaotropics on the structure of the water and on the binding mechanism to DPPC interface.

Adsorption isotherms revealed that monovalent cations are weakly bound to PCs without a specific affinity as the association constant depends on the charge of the metal ion in the order: $Na^+ < Ca^{2+} < La^{3+}$ [9]. This agrees with our observation that all the monovalent cations behave similarly inducing the same shift in T_p.

In contrast to monovalent cations, selective binding of inorganic monovalent anions with PC membranes has been demonstrated in a wide number of studies [1,3,6,8,11,15, 16,19]. It seems that the association constant increases with increasing the chaotropic tendency of the anion [1,6,8,11,16,19]. Limiting the attention to the anions used in our study, for their increasing tendency to disrupt the water structure and to decrease the hydrogen bonding between the membrane and the surrounding water structure, they are arranged in an Hofmeister series [21,22]: $Cl^- < Br^- < NO_3^- < I^- < SCN^-$. In this connection, Cl^- and SCN^-, i.e., one of the least and one of the most chaotropic, show the lowest and the highest binding constant, respectively (Table V in Ref. [19]). Note that Tatulian reported a rather similar binding constants of 0.15 and 0.20 M^{-1} for Na^+ and Cl^- for egg PC at 25°C [11].

Keeping this in mind, it is very likely that the strongly

solvated cations and the common anion Cl^- mutually rather than independently interact electrostatically with the negative, $-PO^-$, and the positive, $-N^+(CH_3)$, charges that form the dipole present on the polar head of each zwitterionic DPPC molecule, respectively. The reduction of the electrostatic interaction between the headgroups, favoured by the blanketing cloud of counter ions present at 1 M, and the rearrangement of the hydrogen bonds favour the close-packing of the polar zone of the DPPC bilayer. This could explain the increase of the pre-transition temperature of about 2°C in presence of Li^+, Na^+, K^+ and Cs^+ at 1 M in the dispersion medium (Table 1). Such an increase of T_p is in agreement with results reported for DPPC [12,13] and DMPC [5] bilayers.

The anions with modest ability to act as 'water-structure breakers', Br^- and NO_3^-, are adsorbed at the DPPC interface where they produce a negative electrostatic potential [8,11,15,16,19]. It is then likely that the electrostatic repulsion may promote a large surface area per headgroup and a loosening of the interfacial region that could account for the decrease of the temperatures of the pre-transition of DPPC relative to those in buffer and in KCl which, of course, behaves as explained above. NO_3^- is slightly more effective in lowering T_p because of its higher chaotropic ability [21,22]. A reduction of T_p in presence of 1 M KBr is given for DPPC and DMPC dispersions [5,13] relative to the T_p value in buffer.

The largest influence on the thermal properties of DPPC bilayers is exerted by the chaotropic anions. Disrupting dramatically the structure of the water layer at the interface of the bilayers and lowering markedly the strength of the hydrogen bonds [21,22], they significantly bind to the DPPC surface conferring a net negative charge [11,15,16,19]. Moreover, they are better able to penetrate the lipid bilayer than the other anions [19] and locate in the plane of the choline quaternary nitrogen [16]. These properties could be the cause of the observed lack of the DPPC pre-transition, most probably associated with the disruption of the ripples usually observed on the surface of PC liposomes. The disappearance of the pre-transition in presence of 1 M KSCN is also reported for PC bilayers on the basis of DSC measurements [4,5,12,13,17]. Electron microscopic studies on the freeze-fractured surface of dispersions of DMPC with 1 M NaSCN, which have the pre-transition endotherm removed, have shown that the ripples have also been removed [5]. Moreover, the action of the chaotropic is extended to the hydrocarbon region of DPPC bilayers since the electrostatic repulsion may lead to a sufficient increase in the area/polar head that looses the packing between lipid molecules and stabilizes the fluid phase. We note that the effectiveness of chaotropic anions in lowering the DPPC main transition temperature, T_m, is almost the same of their ability to destroy the structure of the water and to lower the hydrogen bond strength [21,22]. Shifts in the T_m-values in presence of 1 M KSCN are reported for PCs [4,5,12,13,17].

4.2. Monovalent ions and DPPC lipid chain order

Consistently with calorimetric [4,5,12], X-ray diffraction [12,13] and Raman [2,7] data, our spin-label EPR measurements show that univalent cations Li^+, Na^+, K^+ and Cs^+ and the anions Br^-, Cl^- and NO_3^- have no effects on the order and on the packing density of the DPPC hydrocarbon chains. This confirms the literature data that the hydrophobic core of the lipid bilayers is not influenced by these ions and that the interaction is limited to the polar zone of the DPPC multilayers [7,9,10,12,13,18].

The chaotropic anions, instead, influence also the hydrocarbon zone of DPPC bilayers, particularly in the gel phase (Figs. 3–5). The possibility that the hydrophobic region of PC bilayers should be indirectly influenced by chaotropic anions at high concentrations is not excluded by Jendrasiak et al. [20].

Moreover, the results reported in the Figs. 4 and 5 indicate, with considerable detail, the existence of a flexibility gradient of increasing mobility on proceeding down the terminal methyl end of the chain for either DPPC/buffer or DPPC/chaotropic systems. This fluidity gradient is similar to that normally found even in the gel state of lipid bilayers, both by spin-label EPR [29–31] and by NMR [30] measurements. Nevertheless, in the presence of chaotropics, the degree of molecular order is progressively reduced until the C-12 position whereas, a slight increase of the order with 16-NSA relative to that of DPPC multilayers in buffer, that does not reach the same degree as 12-NSA, occurs in the gel phase. This behaviour could be due to the fact that the chaotropics increasing the bilayer surface area per headgroups in the gel phase and inducing disorder along the DPPC lipid chains, lead to the formation of rotational isomers or kinks that could reduce the width of the bilayer. In such conditions, it is very likely that the 16-NSA of one monolayer penetrates to some extent the opposing monolayer and a reduction of the degree of its motion occurs. In other words, in the presence of chaotropics, the hydrocarbon tails around the bilayer midplane should be slightly interdigitated in the gel phase. The interpenetration of the chains that we believe to occur could then result from a concomitant increase in the bilayer surface area per headgroups and a decrease in bilayer thickness in the gel phase. As surface adsorbing molecules, the influence of the chaotropics is primarily on the chain packing in the gel phase, where the area per headgroup is lower, and is not so appreciable in the fluid phase.

Some studies have shown that chaotropic anions at 1 M concentration affect the structural parameters of the phosphatidylcholine bilayers [13,14,17]. In fact, recently, Cunningham and Lis [13] indicated that the DPPC *d*-spacing, i.e., the lamellar repeat spacing, is reduced from 62 Å for DPPC/water to 52 Å for DPPC/KSCN systems, respectively. As interpretation of this result, the possibility of fully hydrocarbon chains interdigitation in the gel phase

was given [13,14,17]. However, some results that they give are rather controversial, especially about the sample history and some data reported (compare, e.g., the temperatures of the main transition reported in the Refs. [13,14]). Moreover, Chapman et al. [5] and Cunningham et al. [12] previously reported that the presence of KSCN in solution leads the *d*-spacing of DPPC to increase if compared to bilayers in water. This is in evident contrast with the results in [13]. Our EPR measurements on the lipid chain order permit to exclude the formation of a fully interdigitated gel phase for DPPC/chaotropic systems. EPR with the spin-labeling technique has been extensively used for recognizing interdigitated gel phase because in such a phase the motion of a lipid or stearic acid spin labeled near its terminal methyl (i.e., 16-NSA) would be restricted (or the order increased) to a degree similar to that experienced by a label located closer to the carboxyl group (as in 5-NSA) [31,32]. In other words, the flexibility gradient should be abolished in the interdigitated gel phase [31,32]. This is not the case with our DPPC dispersions preparation. Additionally, a bilayer structure of two opposed monolayers is consistent with the results of Rydall and Macdonald [19] on POPC liposomes. Their ^{2}H- and ^{31}P-NMR spectra, although showing an approximate 10% decrease in the absolute value of the chemical shift anisotropy in the presence of chaotropic anions, retain a line shape consistent with a bilayer arrangement of the lipids.

In conclusion, the chaotropic anions at 1 M concentration do not induce a phase transformation from a normal bilayer structure to a fully interdigitated bilayer in the gel phase of our DPPC dispersions preparation. Two opposed monolayers in which the terminal hydrocarbon tails could be interdigitated or interpenetrated in the DPPC gel phase seem more plausible.

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