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EFFECT OF COUNTERION BINDING AND ALKYL CHAIN LENGTH ON THE PHASE TRANSITION BEHAVIOUR OF DI-*n*-ALKYL PHOSPHATE VESICLES

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This paper describes a fluorescence depolarization and ^{31}P NMR spectroscopic study of the phase transition behaviour of a series of identical and mixed-chain di-*n*-alkyl phosphate vesicles in the presence of different counterions (Na^+, K^+, Me_4N^+, Ca^{2+}). Using *trans,trans,trans*-1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescent probe, the fluorescence polarization (P) was measured for the identical-chain vesicles (Na^+, K^+, Me_4N^+) as a function of temperature. The temperature for the main phase transition (T_m) only responded to variation of the counterion in the case of the longer-chain di-*n*-alkyl phosphates, with T_m decreasing in the sequence $Na^+ > K^+ > Me_4N^+$. This result is rationalized in terms of a decreasing counterion binding, which affects chain ordening in the core of the bilayer. Peak intensities and line widths of the ^{31}P NMR resonances for the bilayer vesicles suggest a more complex phase behaviour, but the overall results are reconcilable with the picture emerging from the fluorescence depolarization experiments. Fluorescence depolarization measurements were also carried out with vesicles formed from the sodium di-*n*-alkyl phosphates and in the presence of various concentrations of Ca^{2+} (0–6 mM) at temperatures above T_m. For both the identical-chain and mixed-chain di-*n*-alkyl phosphate vesicles, a steep increase in P was found between *ca* 1.0 and 1.4 mM Ca^{2+}, indicative of a strong Ca^{2+}-induced ordening of the alkyl chains.

INTRODUCTION

Surfactant aggregation is determined by a complex interplay of different types of intermolecular interactions including those contributed by the solvent.[1,2] The size and shape of the aggregate is primarily dependent on the molecular architecture of the surfactant monomer, but factors such as temperature, pressure and the presence of other solutes also play a distinct role. In fact, surfactant assemblies illustrate well the concept of supramolecular chemistry, i.e. 'the chemistry of the intermolecular bond, covering the structure and functions of the entities formed by association of two or more chemical entities.'[3]

Molecular bilayers formed from phospholipids are among the most important supramolecular structures in biology since they form the structural basis of biological membranes. Interestingly, well defined synthetic surfactants (usually double-chained) also form bilayers which are structurally simpler than phospholipid bilayers. Moreover, these vesicular systems can successfully mimic structural and functional aspects of biological membranes such as (i) the occurrence of phase transitions (e.g. from a gel-like to a liquid-crystalline state),[4] (ii) the capacity to serve as a matrix for reconstitution of membrane proteins,[5] (iii) the capacity to display osmotic activity[6] and (iv) the ability to engage in fusion (merging of the bilayers of two or more vesicles).

In previous studies,[7] we have shown that vesicles formed from di-*n*-alkyl phosphate surfactants can fuse efficiently in the presence of Ca^{2+} ions. Further, we have shown that these types of vesicles are also capable of fusing specifically with model and biological

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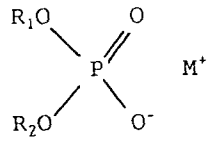
membranes.[8] Since the fusogenic behaviour of di-*n*-alkyl phosphate vesicles is intimately related to the packing of the alkyl chains in the bilayer, we have previously determined phase transition temperatures (T_m) for the main phase transition from a relatively rigidly packed, gel-like state to a more fluid-like, liquid-crystalline state.[9] For a series of vesicle-forming di-*n*-alkyl phosphates $(R_1O)(R_2O)PO_2^-M^+$, with both $R_1 = R_2$ and $R_1 \neq R_2$), the T_m values were found to decrease progressively with decreasing chain lengths (C_{18}–C_{10}) and increasing asymmetry of the alkyl chains.

In this paper we describe the effects of different counterions (Na^+, K^+, Me_4N^+ and Ca^{2+}) on the main phase transition of bilayers formed from a series of symmetric and asymmetric di-*n*-alkyl phosphates. The transitions were monitored by fluorescence depolarization, using *trans,trans,trans*-1,6-diphenylhexatriene (DPH), intercalated in the hydrophobic core of the bilayer. Further, the interaction of the counterion with the phosphate headgroup of the surfactant in the vesicles was studied by ^{31}P NMR spectroscopy. The results indicate and emphasize that the type of counterion is an important structural parameter in determining the physical properties of the present vesicular aggregates.



1 $R_1 = R_2 = n\text{-}C_{10}H_{21}$; $M^+ = Na^+$ (**a**), K^+ (**b**), Me_4N^+ (**c**)
2 $R_1 = R_2 = n\text{-}C_{12}H_{25}$; $M^+ = Na^+$ (**a**), K^+ (**b**), Me_4N^+ (**c**)
3 $R_1 = R_2 = n\text{-}C_{14}H_{29}$; $M^+ = Na^+$ (**a**), K^+ (**b**), Me_4N^+ (**c**)
4 $R_1 = R_2 = n\text{-}C_{16}H_{33}$; $M^+ = Na^+$ (**a**), K^+ (**b**), Me_4N^+ (**c**)
5 $R_1 = n\text{-}C_{10}H_{21}$; $R_2 = n\text{-}C_{14}H_{29}$; $M^+ = Na^+$
6 $R_1 = n\text{-}C_{10}H_{21}$; $R_2 = n\text{-}C_{16}H_{33}$; $M^+ = Na^+$
7 $R_1 = n\text{-}C_{10}H_{21}$; $R_2 = n\text{-}C_{18}H_{37}$; $M^+ = Na^+$
8 $R_1 = n\text{-}C_{12}H_{25}$; $R_2 = n\text{-}C_{14}H_{29}$; $M^+ = Na^+$
9 $R_1 = n\text{-}C_{14}H_{29}$; $R_2 = n\text{-}C_{16}H_{33}$; $M^+ = Na^+$

RESULTS AND DISCUSSION

Monovalent counterions. Fluorescence depolarization

All di-*n*-alkyl phosphates (**1a–c**, **2a–c**, **3a–c**, **4a–c**, **5–9**) used readily form unilamellar vesicles as revealed by cryo- and 'thin-section' electron microscopy.[9] Electron micrographs of vesicles stained with uranyl acetate suggested that the stain causes aggregation and, above a certain concentration, fusion. In all cases the vesicles were prepared by the ethanol-injection method[10] using a standard procedure.[9] Fluorescence depolarization is one of the standard techniques for studying the physical state of a bilayer.[11]

We used *trans,trans,trans*-1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescent probe which readily reports physical changes in the hydrophobic interior of the bilayer.[12] These changes are revealed by alterations of the degree of fluorescence polarization (P), which is calculated from

$$P = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$$

where $I_\parallel$ and $I_\perp$ are the fluorescence intensities detected with the polarizers oriented parallel and perpendicular, respectively, to the direction of polarization of the excitation light. The value of P is determined by the rate of rotation of the rod-like probe molecule, trapped in the bilayer core, which carries the excitation transition moments into changing angular distribution patterns while emission of light takes place. For isotropic rotational diffusion of the probe, $P = 0$. Different values of P correspond to different extents of ordering of the probe, this ordering being substantially higher for the gel-like than for the liquid-crystalline state of the bilayer.

Table 1 gives P values as a function of temperature for a series of vesicles prepared from symmetric di-*n*-alkyl phosphates carrying different counterions (Na^+, K^+, Me_4N^+). Except for **1a–c**, the bilayers undergo a cooperative main phase transition, from which the T_m values can be readily obtained (Table 1). As expected,[9] T_m increases with increasing chain lengths of R_1 and R_2. Most interesting, however, is the observation that T_m is

Table 1. Fluorescence polarizations (P) and phase-transition temperatures (T_m) of vesicles formed from **1a–c**, **2a–c**, **3a–c** and **4a–c**

Amphiphile[a]	P[b]	T_m (°C)
1a (55)	0.34(2), 0.27(15), 0.20(37), 0.17(52)	8[c]
1b (55)	0.27(2), 0.17(8), 0.10(25), 0.08(54)	~8[d]
1c (55)	0.25(2), 0.19(12), 0.13(30), 0.08(52)	~8[d]
2a (55)	0.38(13), 0.35(24), 0.24(30), 0.08(50)	28[e]
2b (55)	0.40(16), 0.27(26), 0.24(30), 0.08(50)	28
2c (55)	0.42(6), 0.35(25), 0.13(30), 0.08(50)	28
3a (70)	0.38(20), 0.34(40), 0.24(50), 0.13(54)	48[e]
3b (70)	0.40(20), 0.34(40), 0.19(50), 0.09(60)	47
3c (70)	0.40(20), 0.25(40), 0.12(50), 0.07(60)	40
4a (70)	0.31(25), 0.21(60), 0.12(70), 0.06(78)	66[e]
4b (70)	0.34(25), 0.25(50), 0.11(60), 0.06(72)	54
4c (70)	0.32(20), 0.18(40), 0.09(50), 0.06(60)	39

[a] The temperature (°C) at which the vesicles were prepared is given in parentheses.
[b] Temperatures (°C) are given in parentheses.
[c] Weakly cooperative transition.
[d] T_m difficult to determine accurately.
[e] See also Ref. 9.

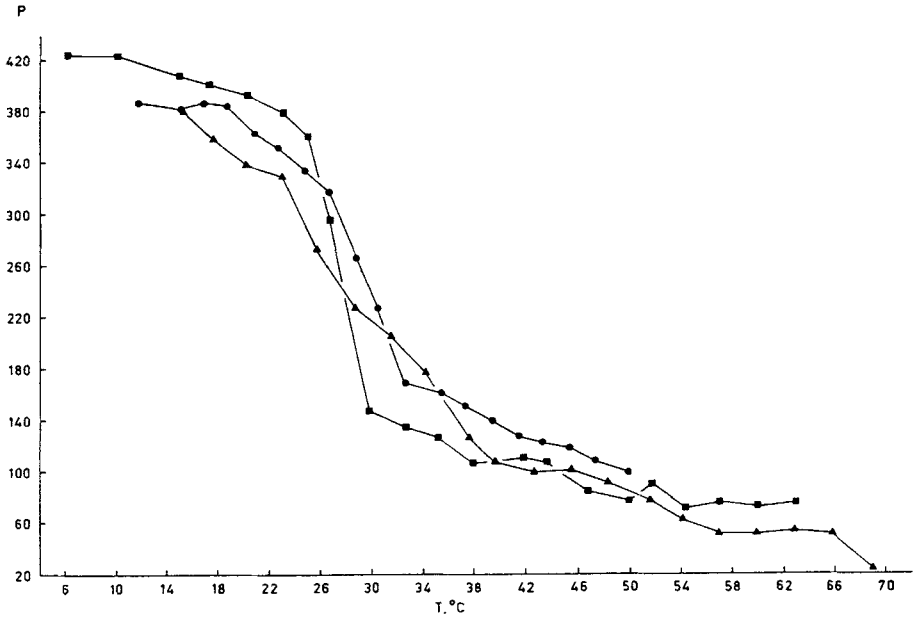


Figure 1. Plot of the fluorescence polarization ($P \times 10^3$) vs temperature for vesicles formed from (•) **2a**, (▲) **2b** and (■) (**2c**)

independent of the counterion for **1a–c** and **2a–c** (Figure 1) whereas for the di-*n*-alkyl phosphates with longer alkyl chains (**3a–c**, **4a–c**) the T_m values decrease in the sequence of counterions $Na^+ > K^+ > Me_4N^+$. The difference in T_m is as much as 27 °C when comparing **4a** and **4c**, while T_m of **4c** is well below that of **3a** (Figure 2). Hence it appears that electrostatic interactions between the phosphate headgroups and the counterions affect the hydrocarbon chain interactions in the core of the bilayer. Just as in the case of cation binding to DNA,[13] we suggest that the counterions bind to the phosphate headgroups with decreasing effectiveness in the order $Na^+ > K^+ > Me_4N^+$. Stronger cation binding reduces headgroup repulsion and allows for tighter alkyl chain interactions, leading to a higher T_m. For the shorter chain amphiphiles **1a–c** and **2a–c** the headgroups are further apart, more strongly hydrated and less apt to bind to counterions.

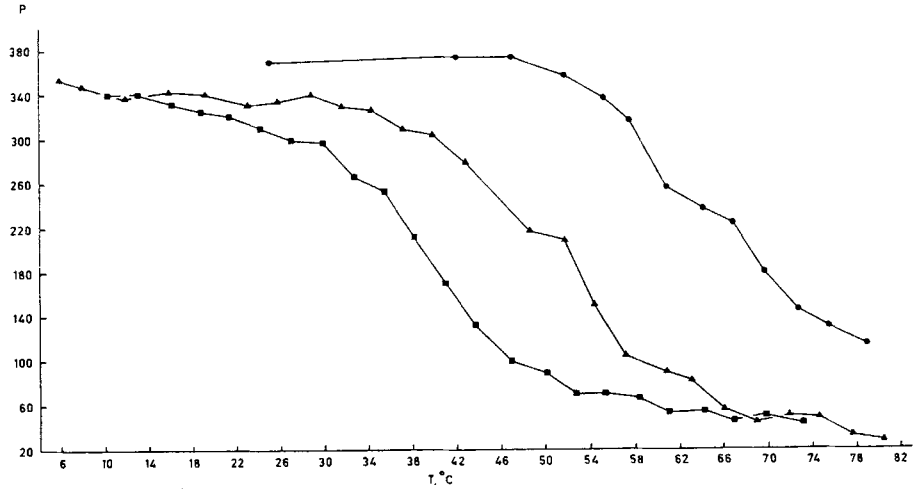


Figure 2. Plot of the fluorescence polarization ($P \times 10^3$) vs temperature for vesicles formed from (•) **4a**, (▲) **4b** and (■) **4c**

Monovalent counterions. ^{31}P NMR spectroscopy

In an attempt to probe the effect of the different counterions on the conformational preferences and dynamics of the surfactant headgroups in the vesicles, we measured peak intensities and line widths (at half-height, $\nu_{1/2}$) of the ^{31}P NMR resonance as a function of temperature, with particular emphasis on the temperature range around T_m. Previous work has shown that ^{31}P NMR spectroscopy is a sensitive tool in the structural analyses of phospholipid vesicles (liposomes)[14] and of aggregates prepared from synthetic surfactants with phosphate headgroups.[7,14] Our results reveal that the phase behaviour of the vesicles used in this study is indeed much more complicated than can be accounted for in terms of a single 'main' phase transition.

Changes in line intensities, small subsequent splitting of the resonances and slow line broadening processes have all been observed under a variety of specific conditions. Although of potential interest, these phenomena can at present not be assigned to a well defined phase behaviour. For example, at temperatures *below* T_m the formation of non-bilayer structures cannot be excluded. Therefore, we restrict our discussion here to those features which are directly relevant to the results obtained from the fluorescence depolarization measurements.

All di-*n*-alkyl phosphate vesicles exhibit a relatively sharp and isotropic resonance (line width *ca* 25–35 Hz) at temperatures above T_m. This result is expected for normal bilayer packing in the liquid crystalline state.[7,15] In the case of the di-*n*-decyl phosphate vesicles **1a–c**, hardly any line broadening is observed on cooling to 2 °C. Therefore, the occurrence of a phase transition ($T_m = 8$ °C, Table 1) is not revealed in a significant change in the rate of headgroup rotation between 8 and 2 °C, and the different counterions have little effect on the phase behaviour of the bilayer.

A different behaviour is found for the di-*n*-dodecyl phosphate vesicles (**2a–c**). For **2a** and **2b**, the line width increases strongly and abruptly on cooling to T_m, and below T_m the resonance disappears completely. Freezing of the headgroup rotation on the ^{31}P NMR time scale also occurs for **2c**, but the line broadening occurs much more slowly. Now the signal disappears completely after cooling from 28 to 5 °C over a period of 7 h. We conclude that the different counterions do not effect T_m of the main phase transition (Table 1), but the headgroup of **2c** apparently displays more rotational freedom below T_m than that of **2a** and **2b**.

Essentially similar behaviour is found for **3a–c**. For **3a** and **3b**, the ^{31}P NMR resonances disappear in the noise below T_m (Table 1) but this effect is strongly retarded for **3c** where it occurs at 25 °C after cooling from 40 to 25 °C over a period of 3 h.

The ^{31}P line broadening processes suggest relatively slow conformational transitions for **4a–c**. Now the ^{31}P resonance does not disappear into the noise below T_m. For **4a**, $\nu_{1/2}$ starts to increase below 50 °C, reaching a value of 150 Hz at 25 °C after a cooling process over a period of 5 h. Broadening also starts below 50 °C for **4b**, but $\nu_{1/2}$ is *ca* 120 Hz after cooling over 6 h from 50 to 20 °C. The Me_4N^+ salt (**4c**) exhibits only a minor line broadening ($\nu_{1/2} \approx 30$ Hz) on cooling from 35 to 10 °C during 5 h.

Despite the complex behaviour of the ^{31}P NMR signals, the overall picture is consistent with that deduced from the temperature dependence of the *P* values.[16] The headgroup rotation is strongly slowed down in the gel-like state of the bilayer, but this retardation is dependent of the alkyl chain lengths and is further modulated by interactions with the counterion. Evidently the latter effects are the weakest for the Me_4N^+ cation.

Effect of Ca^{2+} ions

We made a preliminary study of the effect of Ca^{2+} ions on the phase behaviour of vesicles formed from **2a** and **5–9**. In previous studies[7,17] we have shown that Ca^{2+} ions are capable of inducing aggregation (threshold concentration 1·38 mM) and fusion (threshold concentration 1·73 mM) of di-*n*-dodecyl phosphate (**2a**; DDP) vesicles (at 40 °C) and a mechanism for the fusion process, involving the formation of inverted micellar intermediates, has been proposed. Quantitative studies of Ca^{2+} binding to DDP vesicles[18] indicate that fusion occurs via a local perturbation of the bilayer, where the formation of a (dehydrated) '*trans*-Ca^{2+}–DDPcomplex' triggers the actual merging of the bilayers. Previously[7] we have briefly described the effect of addition of Ca^{2+} ions on the *P* values of DPH-labelled DDP vesicles (pH 7·4) at a temperature (40·2 °C) well above T_m. It was found that the smooth increase in *P* at low Ca^{2+} concentrations turns into a steep and abrupt increase above 1·0 mM of Ca^{2+}. Around 1·4 mM Ca^{2+}, *P* levels off to a constant value of about 0·35 which is typical for a strong alignment of DPH molecules along the alkyl chains of the surfactant in the gel-like state of the assembly. It is clear that binding of Ca^{2+} ions to the phosphate headgroups has a pronounced effect on the alkyl chain packing and that this effect takes place well below the Ca^{2+} threshold concentration for aggregation of the vesicles.

The data suggest that T_m for the DDP–Ca^{2+} complex is shifted to a temperature above 65 °C (T_m for DDP in the absence of Ca^{2+} is 28 °C). This isothermal shift of T_m is in line with similar observations for phospholipid vesicles, e.g. the phosphatidyl–Ca^{2+} complex.[19] Hence it is suggested that Ca^{2+} binding brings the headgroups in the vesicles more closely together and reduces the dynamics of the headgroups. This effect is than propagated into the bilayer, leading

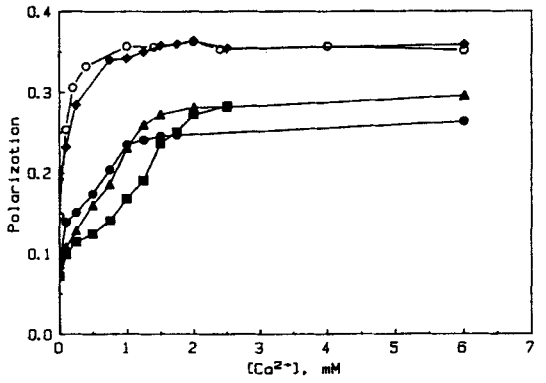


Figure 3. Effect of Ca^{2+} on the fluorescence polarization (P) of DPH-labelled vesicles formed from (•) **5** (35 °C), (▲) **6** (40 °C), (■) **7** (50 °C), (○) **8** (42 °C) and (◆) **9** (59 °C) (pH 7·4)

to a high degree of ordering of the alkyl chains. A detailed discussion of the phase behaviour, particularly at higher Ca^{2+} concentrations will be presented elsewhere.[20]

Vesicles formed from the mixed-chain di-*n*-alkyl phosphate surfactants **5–9** all show similar large increments of P on addition of Ca^{2+} ions (Figure 3). The most pronounced increase in P is found for **6–8** ($\Delta P = 0{\cdot}208 \pm 0{\cdot}003$) and a smaller increase ($\Delta P = 0.171 \pm 0{\cdot}005$) is found for **5** and **9**. Comparison of the increment in P for DDP (**2a**, $\Delta P = 0{\cdot}23$) with that for the vesicles formed from **5** (same total number of carbon atoms; $\Delta P = 0{\cdot}176$) suggests that the increase in chain ordering on Ca^{2+} binding is smaller for the mixed-chain surfactant vesicles. This may be a consequence of interdigitation of the non-identical chains across the centre of the bilayer, but more definite conclusions can only be drawn after more detailed studies of the phase behaviour of these vesicular systems.

EXPERIMENTAL

General. The syntheses of **1a**, **2a**, **3a**, **4a** and **5–9** have been described previously.[9] The potassium and tetramethylammonium salts were prepared analogously from the corresponding analytically pure di-*n*-alkyl hydrogenphosphate using potassium ethoxide in ethanol and tetramethylammonium hydroxide in methanol, respectively. After evaporation of the solvent, the salts were crystallized from anhydrous ethanol. In all cases the vesicles were prepared by the ethanol-injection method[9,10] at a temperature above T_m (Table 1) using HEPES–acetate buffer solutions at pH 7·4.

Fluorescence depolarization. These measurements. were performed as described before.[9] Particularly around T_m, P values (±0·01) were determined over small temperature steps (3–5 °C). At each temperature, the solution was equilibrated for at least 15 min. Longer equilibration times (up to 60 min) did not result in significantly different P values. The amphiphile concentration was 5×10^{-5} M. The data shown in Figure 3 were obtained for fresh vesicle solutions (final surfactant concentration 5×10^{-5} M). The required amount of an aqueous solution of $CaCl_2$ was added to these solutions and measurements were started after 15 min of equilibration at a temperature (Figure 3) above T_m.

^{31}P NMR spectroscopy. These measurements were carried out (at 121·4 MHz on a Varian VXR 300 spectrometer) under carefully controlled conditions as described previously.[9] Line-width measurements were always initiated at the highest temperature and were performed in temperature steps of 5 °C. At each temperature the sample was equilibrated over a period of 15 min.

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