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A calorimetric study of the influence of divalent cations on the thermotropic behaviour of some phosphatidylcholines

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Abstract

The gel to liquid-crystal phase transition of different phosphatidylcholine liposomes has been studied by differential scanning calorimetry in the presence of divalent cations (Ca^{2+}, Zn^{2+}, Cu^{2+}) of relevant biological interest. The calorimetric experiments were carried out at different ion concentrations.

The effects on the transition temperature were observed as shifts in the calorimetric peaks towards higher values, even if their magnitudes were lower than those reported for charged lipids. A very small effect on the enthalpy variation was shown to be associated with this transition.

We tried to relate the modifications of the thermotropic behaviour of the examined phospholipids with the ionic radius, taking into account also the area per molecule occupied by phosphatidylcholines; the calorimetric results support the idea that the ion–lipid interaction with formation of the complexes is a function of the area occupied by the lipid head group.

INTRODUCTION

In biological systems some divalent cations such as Ca^{2+} and Mg^{2+} are involved in regulation of numerous cellular functions including stimulus–contraction and stimulus–secretion couplings, intermembrane cellular interactions, membrane fusion and fluidity, and enzyme activation [1]. Moreover, Cu^{2+} is an essential constituent of some enzymes such as cytochrome oxidase, superoxide dismutase and dopamine β-hydroxylase [2–4], Zn^{2+} is present in carboxypeptidase, carbonic anhydrase, superoxide dismutase, and RNA and DNA polymerase [5–8].

The uptake of ions by biological membranes is due in part to binding sites on the phospholipid bilayer [9]. As phosphatidylcholine (PC) is generally a

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major component of the total phospholipid occurring in natural membranes, it is very interesting to study the interaction of ions with this phospholipid.

One way to study the effect exerted by ions on biological membranes is to make use of membrane models, usually liposomes of dipalmitoylphosphatidylcholine (DPPC), and to perform differential scanning calorimetry (DSC) measurements on mixtures of lipids and ions. Aqueous dispersions of phospholipids display thermotropic behaviour, the well-known gel to liquid-crystal phase transition associated with the melting of hydrocarbon chains and the subsequent increase in rotational and translational motion of lipid chains [10,11]. The presence of ions could modify substantially this behaviour by changing the two main parameters obtainable: the transition temperature (T_m) and the variations in the enthalpy (ΔH) associated with the calorimetric peak of these phase transitions.

The variations of the thermodynamic parameters could indicate the interaction of a component with the lipids, interaction that is related to the lipid structure, i.e. the polarity of the head groups, the alkyl chain length and the degree and type of unsaturation.

For this purpose we investigated the effect of some divalent cations on the molecular species of phosphatidylcholines present in biological membranes, having a different fatty acid composition.

MATERIALS AND METHODS

Chemicals

Synthetic L-α-dipalmitoylphosphatidylcholine (DPPC) and L-α-dimyristoylphosphatidylcholine (DMPC) were purchased from Fluka Chemical Co. (Buchs, Switzerland); L-α-dioleoylphosphatidylcholine (DOPC) and L-α-phosphatidylcholine-β-oleoyl-γ-stearoyl (OSPC) were purchased from Sigma. Solutions of lipids were chromatographically pure as assessed by two-dimensional thin layer chromatography (TLC). The phosphorus content of the phospholipids was essayed as inorganic phosphate by the analytical procedures previously reported [12].

Stock solutions of $CaCl_2$, $CuCl_2$ and $ZnSO_4$ in KNO_3 (0.1 M; pH 5.0) were prepared and the metal ion contents were checked by ethylenediaminetetraacetic acid (EDTA) titration.

Preparation of liposomes

Aqueous dispersions of pure lipids were prepared by the following procedure. Lipid solutions were prepared in $CHCl_3$–CH_3OH (1 : 1, v/v). The solvent was removed at 30 °C on a rotary evaporator in a stream of nitrogen and the resulting film was lyophilized for 3 h.

Liposomes were prepared by adding aqueous solutions of KNO_3 (0.1 M) or different aliquots of the salt solutions (0.1 M in KNO_3) to the lyophylized lipid film to obtain the required ion concentrations.

The pH values of the solutions were measured: 6.0 for Ca^{2+}; 5.0 for Zn^{2+} and 3.5 for Cu^{2+}. These pH values are all in the range within which the PCs are present as neutral phospholipids [13].

A mathematical simulation was performed to obtain information on the ionic species present in the lipidic dispersion at the pH at which the experiments were carried out by using the model reported in the literature (see ref. 14). For Zn^{2+} and Cu^{2+} at all the ion concentrations used, we obtained evidence that the pH levels at which the calorimetric measurements were performed were such that the formation of hydroxy species was avoided.

Liposomes were prepared by adding the KNO_3 solution to the film, at a temperature greater than that of their gel–liquid-crystal phase transition to allow full hydration of the samples. The samples were vortexed twice for 1 min at 70 °C and then shaken for 3 h at 70 °C in a water bath to homogenize the dispersion. Afterwards, 120 μl aliquots (5 mg) of each sample (for the experiments with Ca^{2+} and Zn^{2+} ions) were transferred to and sealed in aluminium pans and submitted to DSC analysis; for the experiments with Cu^{2+} ions, 70 μl (3 mg) were transferred to and sealed in a glass pan to avoid oxidation of the aluminium pan in the presence of Cu^{2+}.

Differential scanning calorimetry

Differential scanning calorimetry measurements were performed with a Mettler TA 3000 calorimeter, equipped with a DSC 30 cell and a TC 10 processor. The samples were analysed by using heating and cooling rates of 2 °C min^{-1}, in the temperature range between −40 and 70 °C (depending on the phospholipid studied), after an isothermal period of 15 min at the starting temperature. Sensitivities of 1.71 mW were used; the same KNO_3 solution was used in the reference pan.

Each sample was heated and cooled at least four times through the lipid phase-transition region to ensure reproducibility of the observed behaviour. Palmitic acid was employed to calibrate the temperature scale and the ΔH. Enthalpy changes were calculated from peak areas.

After the calorimetric runs, the contents of the pan were removed and aliquots were taken for phosphate analysis to determine the amount of phospholipid in the pan.

RESULTS AND DISCUSSIONS

We have studied the gel to liquid-crystal phase transition for four different neutral PCs (DPPC, DMPC, DOPC and OSPC) in the presence and absence of Ca^{2+}, Zn^{2+} and Cu^{2+}.

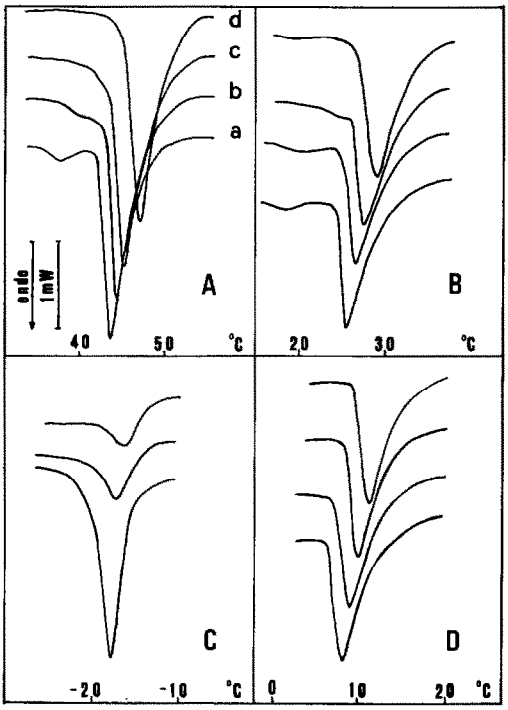

Fig. 1. Typical DSC heating curves of the hydrated lipids DPPC (A), DMPC (B), DOPC (C) and OSPC (D) in the presence of Ca^{2+} at different molar concentrations: curve a, 0; curve b, 0.056; curve c, 0.227; curve d, 5.

Figures 1–3 show the calorimetric curves, in heating mode, for the pure lipids DPPC (A), DMPC (B), DOPC (C) and OSPC (D) as well as for the lipids in the presence of increasing amounts of metal ions. The thermodynamic data (T_m and ΔH) obtained from the DSC curves of the pure lipids are in good agreement with the literature values [15–17].

The DSC curves show that the PC liposomes experience a shift to higher T_m values, without variation in the peak areas, with increasing ion concentrations (see also Table 1). Consequently the associated enthalpy changes (ΔH) remain nearly constant. The only PC showing a different behaviour was DOPC: with increasing Ca^{2+} concentration, a reduction in the peak area was observed in addition to the shift in T_m, whereas no significant area variations were observed in the presence of zinc or copper ions.

Figures 4–6 show the above-mentioned shifts as transition temperature differences (ΔT_m) with respect to the value obtained for the pure lipids. The effectiveness of the interaction of the ions seems to be as follows: DPPC > DMPC > DOPC > OSPC for Ca^{2+}; DPPC > DMPC > OSPC > DOPC for Zn^{2+}; DPPC > DMPC > OSPC > DOPC for Cu^{2+}.

The experiments carried out demonstrate that positively charged ions exert an effect on the gel to liquid-crystal phase transition of neutral lipids:

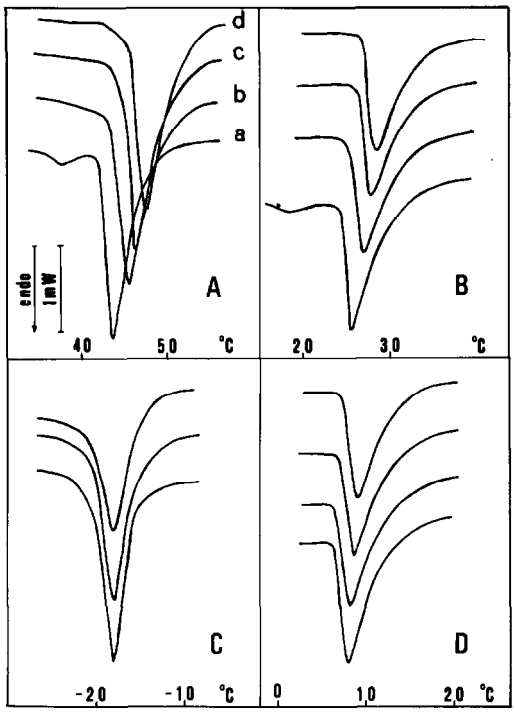

Fig. 2. Typical DSC heating curves of hydrated lipids DPPC (A), DMPC (B), DOPC (C) and OSPC (D), in the presence of Zn^{2+} at different molar concentrations: curve a, 0; curve b, 0.056; curve c, 0.227; curve d, 5.

increasing the cationic concentration shifts T_m towards higher values. This effect is interpreted as a change in the lipid layer structure; it becomes more constrained, suggesting that a stabilization of the lipid structure is occurring.

The interaction of metal ions with charged and uncharged lipids has been studied using different techniques (DSC, NMR, fluorescence) and theories of the mechanism of such an interaction have been reported [1,18–24]. Many of these studies concerned only the system formed from Mg^{2+} and Ca^{2+} and charged or neutral lipids; only a few considered Cu^{2+} and Zn^{2+}, rather than Ca^{2+}, and neutral phospholipids.

The interaction of bivalent cations with neutral phospholipids, which influences the bilayer packing and the structure, is less than that observed for acidic phospholipids (see also the binding constant for the Ca^{2+} ion on PC and phosphatidylserine (PS) [25]) but it is sufficiently strong to detect from the calorimetric data presented here that the ions studied have an effect on the bilayer structure in a hydrated form.

The ion binding to the phospholipid seems to depend on the ion type, ion concentration and lipid hydrocarbon chain. Examining the last variable, we found that our results for Ca^{2+} agreed with the work of Lis et al. [23]: the binding of Ca^{2+} to various PCs increases in the sequence DPPC $\geqslant$ DMPC >

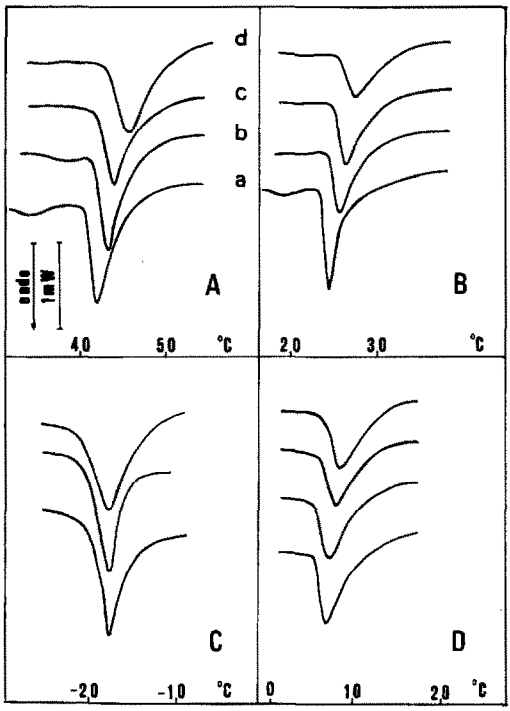

Fig. 3. Typical DSC heating curves of hydrated lipids DPPC (A), DMPC (B), DOPC (C) and OSPC (D), in the presence of Cu^{2+} at different molar concentrations: curve a, 0, curve b, 0.056; curve c, 0.227; curve d, 5.

DOPC; similar effects were found also for Zn^{2+} and Cu^{2+}. The importance of the lipid hydrocarbon chain in the ionic interaction with the polar head groups can be understood by considering the monolayer experiments carried out by Demel and co-workers [26,27] and Lund-Kantz et al. [28] (see also ref. 29). They reported the area per molecule for synthetic diacylphosphatidylcholine and it appears that decreasing the chain length (passing from DPPC to DMPC) or introducing a double bond in one (OSPC) or both of the aliphatic chains (DOPC) increases the value of the area per molecule in the order DPPC < DMPC < OSPC $\leqslant$ DOPC. These findings fit well with our calorimetric results (see the above-reported scale of ion interactions with the PCs): on increasing the area per molecule it becomes more difficult for the cations to find a pair of phospholipid head groups at the right distance to form a binary complex.

The results can also be considered from another point of view; by comparing the effect of the cations with their ionic radius (Cu^{2+}, 0.69 Å; Zn^{2+}, 0.74 Å; Ca^{2+}, 0.99 Å) and their cumulative constant, $\beta[M^{2+}H_2L^-]$, with respect to phosphoric acid: (Cu^{2+}, 1.3; Zn^{2+}, 1.2; Ca^{2+}, 0.6, values obtained at ionic strength $I = 0.15$ in KNO_3 and $t = 37\,^\circ C$ [30]). It seems that the largest ion (Ca^{2+}) exerts a greater effect on the molecule with the

TABLE 1

Main transition peak temperature, T_m (°C), and main transition enthalpy changes, ΔH (kcal mol^{-1}), of PC dispersions for different molar ion concentrations. Mean values obtained from at least four DSC heating curves are given

		Ion concentration (M)						
		0.0	0.01	0.028	0.056	0.10	0.227	0.50
Ca^{2+}/DPPC	T_m	42.0	43.1	43.5	43.8	44.2	44.9	46.4
	ΔH	8.4	8.5	9.4	8.8	8.4	9.5	9.7
Ca^{2+}/DMPC	T_m	24.8	25.5	25.9	26.3	26.7	27.4	28.8
	ΔH	6.1	5.7	5.8	5.5	5.4	6.4	7.6
Ca^{2+}/DOPC	T_m	−18.6	−18.0	−17.7	−17.5	−17.1	−16.3	
	ΔH	7.6	5.2	4.8	2.2	2.0	1.6	
Ca^{2+}/OSPC	T_m	7.3	7.5	7.8	8.1	8.4	9.1	10.5
	ΔH	5.3	5.8	5.6	5.3	4.9	5.1	5.0
Zn^{2+}/DPPC	T_m	42.0	43.8	44.3	44.8	45.1	45.6	46.6
	ΔH	8.5	8.6	9.3	9.4	9.7	8.6	8.9
Zn^{2+}/DMPC	T_m	24.8	25.4	26.0	26.3	26.7	27.1	27.7
	ΔH	6.1	5.3	5.8	6.1	6.2	5.9	5.8
Zn^{2+}/DOPC	T_m	−18.6	−18.7	−18.5	−18.6	−18.7	−18.6	
	ΔH	7.6	7.1	7.8	7.7	8.0	7.9	
Zn^{2+}/OSPC	T_m	7.3	7.5	7.7	7.8	8.0	8.2	8.6
	ΔH	5.3	5.6	5.8	5.5	5.4	5.0	5.1
Cu^{2+}/DPPC	T_m	42.0	42.8	43.1	43.5	43.8	44.6	45.8
	ΔH	8.5	8.2	7.4	8.5	8.4	6.7	8.3
Cu^{2+}/DMPC	T_m	24.8	25.1	25.8	26.1	26.5	27.2	28.4
	ΔH	6.1	5.7	5.6	5.4	5.7	5.6	5.0
Cu^{2+}/DOPC	T_m	−18.6	−18.4	−18.3	−18.0	−17.9	−17.9	
	ΔH	7.6	7.2	7.8	7.3	7.0	7.5	
Cu^{2+}/OSPC	T_m	7.3	7.6	7.8	8.0	8.2	8.5	8.9
	ΔH	5.3	5.5	5.4	5.4	5.1	5.0	5.2

largest area, DOPC, than the smaller Zn^{2+} and Cu^{2+} ions; this behaviour corresponds to that shown in Figs. 4–6. A similar trend was also found for OSPC, whereas for DMPC, Zn^{2+} and Cu^{2+} have an effect similar to that of Ca^{2+}, and for DPPC, Zn^{2+} shows an effect higher than that of Ca^{2+}, probably due to its higher binding constant.

The interaction of the divalent cations tested here with the phosphorylcholine polar group is represented by the increase in the transition temperature, but when a divalent cation is added to a PC–water system, the ions are adsorbed onto the polar head groups, introducing a long-range coulombic repulsion between the phospholipids forming the bilayers, as well as between the different bilayers. As a consequence of this electrostatic repulsive interaction between the lipids belonging to the same bilayer, a destabilization of the structure should be obtained, leading to a decrease in the transition temperature caused by the less restricted packing of the lipid

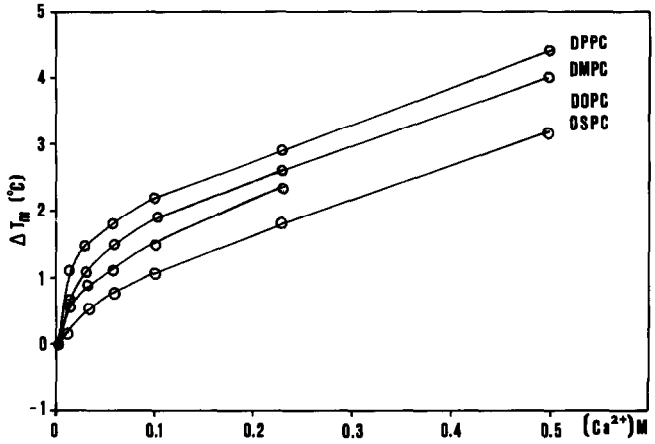

Fig. 4. Transition temperature differences, ΔT_m (°C) (average of at least 4 runs), in heating mode, of hydrated PCs in the presence of Ca^{2+} at the molar concentrations 0, 0.01, 0.028, 0.056, 0.10, 0.227 and 0.5.

molecules—the opposite of the results found. A thermodynamic model has recently been developed to rationalize the calorimetric results [31]. It takes into account the attractive energy among the adsorbed ions and the anionic site of the lipid head-group in addition to the reorganization energy of the surrounding lipid head groups; a correspondence between the experimental and theoretical results was obtained [31].

The suggested mechanism is similar to that proposed by Aruga and co-workers [20,21] for the Ca^{2+}/DPPC system, where a positively charged lipid–Ca^{2+} complex is formed by the Ca^{2+} binding to the negatively

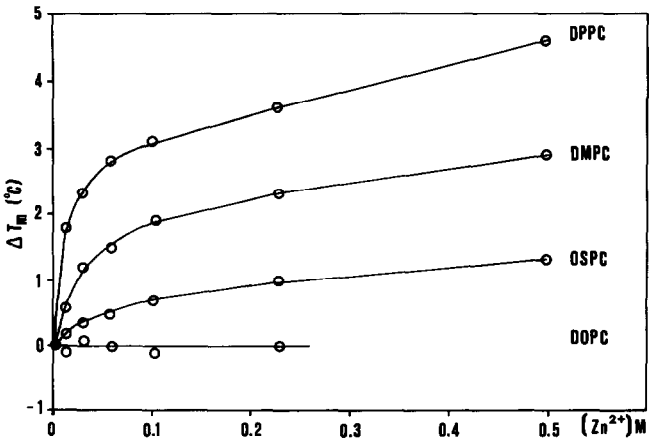

Fig. 5. Transition temperature differences, ΔT_m (°C), (average of at least 4 runs), in heating mode, of hydrated PCs in the presence of Zn^{2+} at the molar concentrations 0, 0.01, 0.028, 0.056, 0.10, 0.227 and 0.5.

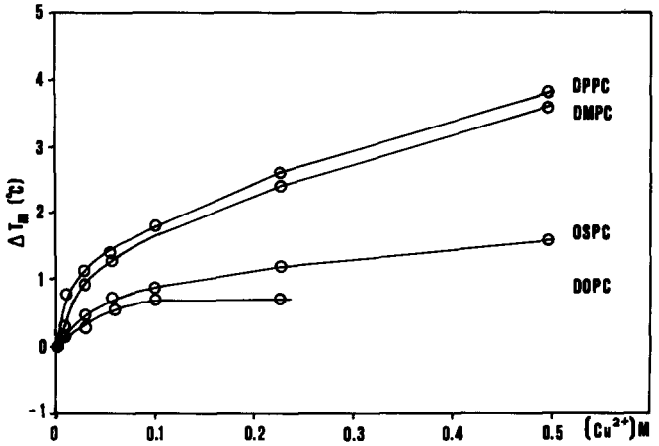

Fig. 6. Transition temperature differences, ΔT_m (°C), (average of at least 4 runs), in heating mode, of hydrated PCs in the presence of Cu^{2+} at the molar concentrations 0, 0.01, 0.028, 0.056, 0.10, 0.227 and 0.5.

charged group of the zwitterionic polar group of DPPC. An electric field shielded by the electric double layer of small ions in the solvent, both perpendicular to the membrane and in the same plane, would be produced. The zwitterionic lipid molecules are then reorganized by the electric field in the plane of the membrane, to permit the negatively charged part to be oriented and bound to Ca^{2+}. The binding of Ca^{2+} to the polar head group of the PCs then induces long-range interaction in the polar region, causing a contraction of the hydrocarbon chain packing throughout the DPPC membrane.

These models were applied to the DPPC–Ca^{2+} system, but their application could be extended to other systems where divalent cations are present in aqueous medium; the considerations concerning the stabilizing effect exerted by positive ions on the PC bilayers should still be valid.

Taking into account the data reported above, i.e. the area per lipid molecule, the ionic radii, the equilibrium constants, the mathematical model and, obviously, the present calorimetric results, we can explain the effect of the ions on the PCs in the following manner.

The Ca^{2+} binding on the phosphate group of the "large" DOPC can be explained in terms of a bridge formation between two lipid molecules; this is supported by the reported 1 : 2 metal ion–phospholipid stoichiometry for this ion [32,33]. A similar explanation can be assumed for the other smaller lipids (lower area per lipid molecule). Therefore, when Ca^{2+} is present the structure of the bilayer becomes more packed and the ΔT_m increases. Cations with a small radius can be expected to bind better with the DOPC phosphate group but are not able to make bridges, thus behaving as univalent cations. Therefore in the presence of Zn^{2+} and Cu^{2+}, the lipid packing is less affected by ion binding and minor or non-existent T_m

variations are observed, even if their equilibrium constants are higher. The combination of all these variables causes some apparent irregularities in their effects with respect to those predicted by the model.

Examining the effect of the cations on OSPC, which has a smaller area per lipid molecule than DOPC, the results are similar, but with smaller differences in ΔT_m. This is because the smaller area of OSPC allows the Zn^{2+} and Cu^{2+} ions to bind better to the polar heads of the lipid molecules, attaining nearly the same bridging effect as Ca^{2+}. A similar effect is observed for DMPC, where the ΔT_m values approach each other for all three cations; this is also a result of the smaller area of the lipid, so the small ions are now favoured in the binding to phosphate groups, taking advantage also of their higher equilibrium constant.

For DPPC, the ΔT_m curve for Zn^{2+} overtakes that of Ca^{2+} and Cu^{2+}. This effect can be explained by considering that the polar head groups of the phospholipids are now close enough for the small Zn^{2+} to exert a greater effect, which is also related to its higher affinity for the phosphate group. All the ΔT_m curves shift progressively towards higher values because the structure of the lipid goes from a fairly loosely packed (DOPC) to a more tightly packed (DPPC) one, becoming even more tight when divalent cations bind to the bilayer surface, as demonstrated by the higher melting point.

Our studies on the interaction of divalent cations with pure phospholipid vesicles, as a model membrane system, should represent a basis for the understanding of their effects in more complex biological membranes. In fact, while other investigations have demonstrated that the binding of divalent cations to biological membranes affects some structural and functional features (see ref. 34 and references cited therein) this work constitutes a further contribution by calorimetric methods to the investigation of the ability of divalent cations to bind onto membrane surfaces, thus modifying their structure and properties.

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