Biochimica et Biophysica Acta, 778 (1984) 17–24
Elsevier

BBA 72300

ESR STUDY OF THE INTERACTION OF MANGANOUS IONS WITH ZWITTERIONIC PHOSPHOLIPIDS AND THEIR MIXTURES WITH CHOLESTEROL

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(Received March 30th, 1984)

Key words: Phospholipid; Mn^{2+}-phospholipid interaction; ESR; Cation-lipid interaction; Sphingomyelin; Phase transition

The adsorption of manganous ions to dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine (DPPE) and sphingomyelin in water dispersions was studied by using ESR spectrometry. The adsorption to phospholipids with phosphorylcholine polar headgroups was found to be greater in the gel phase than in the liquid-crystalline phase. The change of adsorption at the main phase transition of these lipids can be described by the Gouy-Chapman theory of the diffuse double-layer. Only a low adsorption without any abrupt change at the main phase transition was observed for DPPE. Cholesterol decreased the adsorption to DPPC and sphingomyelin in the gel phase. From our results, a conclusion can be drawn that the adsorption of manganous ions to zwitterionic phospholipids is determined both by intermolecular interactions of their polar headgroups and by the state of their aliphatic chains.

Introduction

Divalent cations influence the properties of biological membranes in many ways. Though a number of their effects may be caused by specific interactions with membrane proteins, some of their effects may originate from more general interactions with the phospholipid part of the membrane surface.

In recent years, the properties of phospholipid bilayers and monolayers have been widely studied, but yet there remains much uncertainty about the precise mechanisms of their interactions with ions. The generally recognized arrangement of phospholipids as bilayers with polar headgroups oriented at the lipid/water interface means that an electrostatic field will extend out into the surrounding water. The simplest treatment of interactions of ions with such surfaces is the Gouy-Chapman theory of the diffuse double-layer supplemented by the Stern equations [1]. McLaughlin and co-workers [2–4] experimentally confirmed the relevance of this theory for description of ion binding and ζ-potentials when several monovalent and divalent ions adsorbed to some anionic and zwitterionic phospholipids. A similar conclusion was reached by Puskin and Coene [5] who examined the adsorption of manganous ions to anionic phospholipids, though some difficulties remain.

In the present paper, we examine the interaction of divalent manganous ions with water dispersions of zwitterionic phospholipids, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and their mixtures with cholesterol, as a function of temperature. Manganese-phospholipid association was determined by ESR spectrometry.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine.

Materials and Methods

Dipalmitoyl-L-α-phosphatidylcholine (DPPC), dipalmitoyl-L-α-phosphatidylethanolamine (DPPE), and bovine sphingomyelin were purchased from P-L Biochemicals, and since they yielded only a single spot in one-dimensional thin-layer chromatography in chloroform/methanol/water (65 : 25 : 4), they were used without further purification. Cholesterol was purchased from Merck, Darmstadt. All lipids were stored dry in the dark at −20°C. $MnCl_2$ of analytical grade was obtained from Labora, n.p.

Water dispersions of phospholipids were prepared as follows. A chloroform solution of the lipid was dried down under vacuum. Then it was transferred into a capillary with an inner diameter of 2.5 mm. Traces of the solvent were removed by pumping in a high vacuum for about 1 h. The proper aqueous solution of $MnCl_2$ was added into each capillary, which was immediately sealed. Before measurements, each sample was sonicated with an ultrasonic generator at 20 kHz for 20 min in a water-bath at temperatures slightly higher than the main phase transition temperature of the lipid. The generator was tuned to give the maximum cavitation. Then the samples were recycled from −20 to +60°C for several times.

ESR measurements were performed on an X-band spectrometer constructed by Dipl.-Ing. Štefan Šurka at the Department of Experimental Physics at the Faculty of Mathematics and Physics of the Komensky University. Each spectrum in the temperature interval 25–80°C was determined relative to a standard solution of $MnCl_2$ in water. The temperature was monitored with a digital millivoltmeter NR 30 in conjunction with a thermocouple taped to the sample tube. Accuracy in temperature readings was ±0.5°C. Samples and standards were always equilibrated for 10 min before measurements were carried out. Each value was determined as an average of three measurements.

The association of manganous ions with phospholipids was determined by ESR spectrometry. Mn^{2+} have a $3d^5$ electron configuration and in a water solution, they exist as high-spin complexes with five unpaired electrons, the resultant electron spin being $S = 5/2$. It can be shown [6,7] that the zero-field-splitting relaxation leads to three Lorentzian lines representing the $|\pm 5/2\rangle \leftrightarrow |\pm 3/2\rangle$, $|\pm 3/2\rangle \leftrightarrow |\pm 1/2\rangle$, and $|-1/2\rangle \leftrightarrow |+1/2\rangle$ transitions.

The ^{55}Mn possesses a nuclear spin $I = 5/2$, and the electron-nuclear spin interaction results in splitting of the above-mentioned three lines into six hyperfine lines. For aquated manganous ions, the zero-field-splitting is very small as compared to the hyperfine splitting. The three zero-field lines will overlap and we can monitor only six hyperfine lines with a width of about 0.0025 T at a room temperature (Fig. 1), each of which is a superposition of the three Lorentzian lines, having in general different widths and intensities.

In a water solution, the Mn^{2+} exist as hexaquo-complexes $[Mn(H_2O)_6]^{2+}$ with an octahedral symmetry. If the precise geometry of such a hexaquo-ion is perturbed by binding to a ligand, the zero-field-splitting becomes more complicated. New absorptions and greater splittings of energy levels may arise. Apart from new absorptions, it leads to a greater splitting of the overlapping zero-field lines resulting in a broadening of the hyperfine lines up to approx. 0.1 T. Practically, it means that the spectrum of bound manganous ions will not be detectable under conditions for monitoring the spectrum of free manganous ions. For complexing of Mn^{2+} with zwitterionic phospholipids, this broadening is not so dramatic, the linewidths being about 0.01 T [8], but this is sufficient to make the spectrum of those with phospholipid-associated ions vanish in conditions for detecting free hexaquo-ions (Fig. 2).

It was found that intensities of characteristic ESR signals of free manganous ions are linear

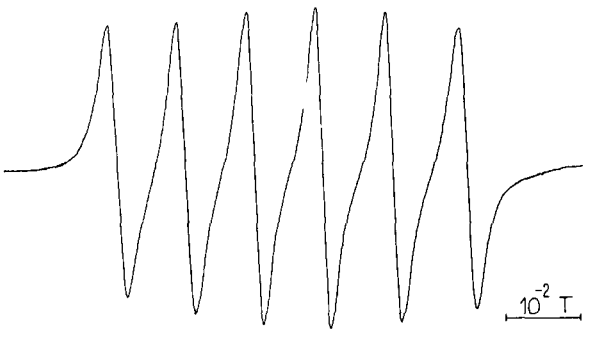

Fig. 1. ESR spectrum of 0.01 M $MnCl_2$ in water at 25°C.

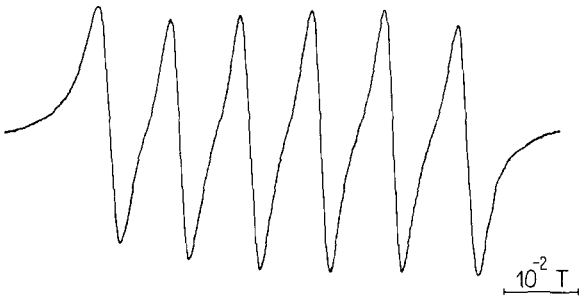

Fig. 2. ESR spectrum of 0.01 M $MnCl_2$ in a water dispersion of DPPC (1.43 M) at 25°C.

functions of their concentration in the range $(10^{-3}$–$10^{-2})$ M [9]:

$$I(\Delta B)^2 = k_1[Mn^{2+}]_f \tag{1}$$

where $[Mn^{2+}]_f$ is the concentration of free manganous ions and k_1 is a constant. As an intensity characterizing parameter we used the expression $I(\Delta B)^2$, where I is the amplitude and ΔB the width of the fourth spectral line. The intensity of the fourth line measured in this way is in general the average of all six lines [10].

Manganese-phospholipid association was characterized by an apparent association constant K_a defined as:

$$K_a = K_i \exp\left\{-\frac{2e\phi_0}{kT}\right\} \tag{2}$$

where ϕ_0 is the electrostatic potential at the lipid/water interface, K_i is the intrinsic association constant which does not depend on the potential ϕ_0, T is the absolute temperature, k is the Boltzman constant, e is the electronic charge. The association constant was estimated from the ratio of bound and free manganous ions [11–13]:

$$K_a = \frac{\beta[Mn^{2+}]_b}{[Mn^{2+}]_f[P]_0} \tag{3}$$

where $[Mn^{2+}]_b$ and $[Mn^{2+}]_f$ are concentrations of bound and free ions, respectively, $[P]_0$ is the total phospholipid concentration and β is a factor taking into account the stoichiometry and the fraction of phospholipid headgroups accessible to Mn^{2+}. Intensities of spectral lines in the presence of phospholipids were calibrated by using standard water solutions of $MnCl_2$ at all temperatures. From Eqns. 3 and 1 it follows:

$$K_a = \beta \cdot \frac{\frac{M_w}{M_s}\left[I(\Delta B)^2\right]_s - \left[I(\Delta B)^2\right]_w}{\left[I(\Delta B)^2\right]_w[P]_0} \tag{4}$$

where M_w is the mass of water in the phospholipid-water dispersion, M_s is the mass of the standard $MnCl_2$/water solution, $[I(\Delta B)^2]_w$ and $[I(\Delta B)^2]_s$ characterize intensities of manganous spectral lines in phospholipid-water dispersions and in standard solutions, respectively.

Results

Temperature dependences of apparent association constants K_a of manganous ions to DPPC, DPPE, DPPC and DPPE mixtures, and sphingomyelin are shown in Fig. 3. For DPPC and sphingomyelin, there was an abrupt decrease of K_a as the lipid passed from the gel into the liquid-

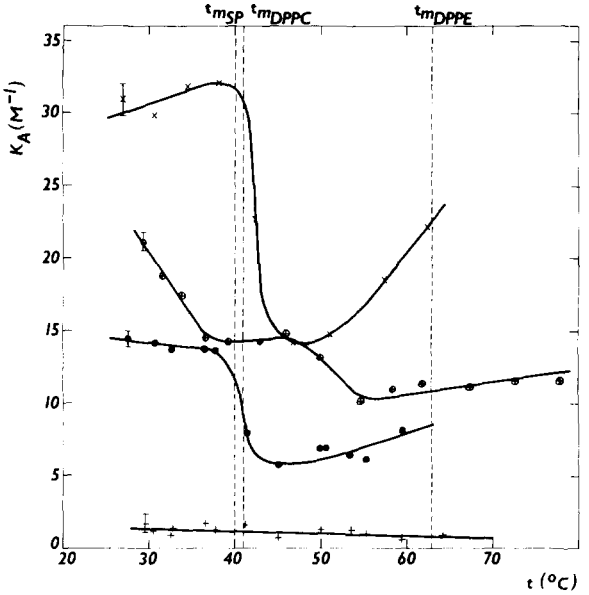

Fig. 3. Temperature dependences of apparent association constants K_a of Mn^{2+} and DPPC (●), DPPE (+), DPPC/DPPE mixture in mole ratio 1:0.658 (⊕) and sphingomyelin (×) in 0.34, 0.46, 0.27 and 0.38 M water dispersions, respectively. The $MnCl_2$ concentration was always 6.7 mM. Main phase transition temperatures of DPPC, DPPE and sphingomyelin, $t_{m_{DPPC}}$, $t_{m_{DPPE}}$, $t_{m_{SP}}$, are indicated.

crystalline state ($t_m = (41 \pm 1)$°C for DPPC, and $t_m = (40 \pm 1)$°C for sphingomyelin [14–18]). For DPPE, no abrupt change of K_a with temperature was observed, and the values of K_a were very low in the whole temperature range investigated. On the other hand, the DPPC and DPPE mixture in mole ratio 1 : 0.658 exhibited qualitatively a similar behaviour as pure DPPC, with a decrease of K_a between main phase transition temperatures of DPPC and DPPE ($t_m = (41 \pm 1)$°C for DPPC, and $t_m = (62 \pm 1)$°C for DPPE [14–18]). By calculations of K_a according to Eqn. 4, we assumed that manganous ions adsorbed to both phospholipids equally.

As DPPC and DPPE have the same palmitic aliphatic chains, the difference in the adsorption of manganous ions must be caused by different chemical composition and intermolecular interactions of their polar headgroups. The low values of K_a for DPPE may be interpreted in two ways:

1. Phosphorylethanolamine groups build a very compact matrix, which is more resistant to hydration than the phosphorylcholine matrix [19–23]. The charge of polar groups on the surface of phosphatidylethanolamine aggregates is therefore probably more neutralized than in the case of phosphatidylcholine.

2. In calculations of the association constant according to Eqn. 4, we have used the parameter $\beta = 1$ for both DPPC and DPPE. This is consistent with the situation when all phosphate groups are accessible to manganous ions, and the stoichiometry of manganese-lipid binding is 1 : 1. In concentrated phospholipid dispersions that we have used, $[P]_0 = (0.3–0.4)$ M, direct interactions between headgroups of phospholipids in neighbouring bilayers may exist and not all phosphate groups must be accessible to manganous ions. In contrast to DPPC, pure DPPE does not form stable vesicles, and it tends to precipitate in a water solution [24]. Therefore, a large proportion of polar headgroups may have been inaccessible to manganous ions in our samples. If this was the case, the low measured values of K_a should be considered as an artefact.

At concentrations of DPPE lower than mole ratio DPPE/DPPC = 0.5 : 1, strong intermolecular interactions of DPPE are eliminated by dilution in such a way that no precipitation occurs, and stable vesicles can be formed from such mixtures [24]. This is in agreement with our finding that the adsorption of manganous ions to a DPPC/DPPE mixture exhibited a qualitatively similar temperature behaviour as for pure DPPC. The observation that the DPPC/DPPE curve does not have binding constants intermediate between those of pure DPPC and DPPE (Fig. 3), but similar to those of pure DPPC, indicates that the affinity of manganous ions to DPPE in mixture, where intermolecular interactions and association between DPPE polar headgroups are lowered or eliminated by dilution, is roughly the same as to pure DPPC.

Apart from the decrease of K_a at the gel to liquid-crystalline phase transition, another decrease of K_a below the main phase transition temperature was observed for the DPPC/DPPE mixture. This change may be connected with the 'pretransition' of the mixture, characteristics of which may be different for pure DPPC, DPPE and their mixture [25]. Though no abrupt decrease in K_a at the pretransition was observed for pure DPPC, the course of its temperature dependence in the gel phase does not agree with predictions of Eqn. 2 (see Figs. 3 and 4). For sphingomyelin, in contrast, Eqn. 2 is in accord with the K_a curve both above and below its main phase transition. As the change in DPPC arrangement at pretransition may have an influence on its affinity to ions, it would be desirable to examine it in more detail in a broader temperature range below the main phase transition.

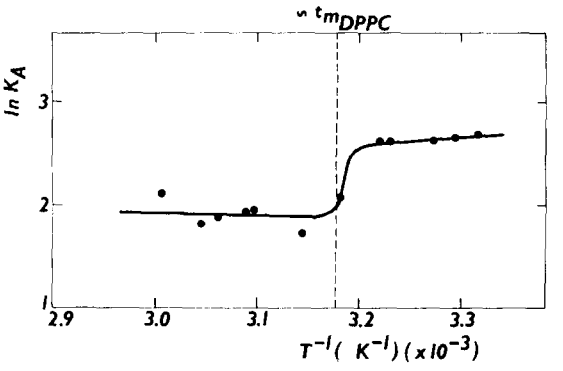

Fig. 4. The Arrhenius plot of apparent association constant of Mn^{2+} and DPPC in 0.34 M water dispersion. The concentration of $MnCl_2$ was 6.7 mM. The main phase transition temperature of DPPC, $t_{m_{DPPC}}$, is indicated.

At present, the generally accepted average orientation of P-N dipoles of polar headgroups of zwitterionic phospholipids is nearly parallel to the bilayer surface in the gel as well as in the liquid-crystalline state [19,23,26,27]. The adsorption of ions to such surfaces can be described by the Gouy-Chapman theory of the diffuse double-layer [1,28–32].

The surface-charge density on phospholipid aggregates with adsorbed manganous ions is:

$$\sigma = \sum_i \frac{z_i e [M_i P_n]_s}{s [P]_{os}} + \sigma_0 \qquad (5)$$

where z_i is the valence of adsorbed ions of i-th species, $[M_i P_n]_s$ is their surface concentration, $[P]_{os}$ is the total surface concentration of phospholipid, s is the surface requirement of one phospholipid headgroup, e is the electronic charge, and σ_0 is an eventual inherent initial surface-charge density.

The adsorption of cations and anions will modify the surface potential of phospholipid aggregates which is nearly equal to zero in the absence of ions [33]. The dependence of surface-charge density σ on surface potential ϕ_0 is expressed by the Graham equation from the Gouy-Chapman theory of the diffuse double-layer:

$$\sigma = \pm \left\{ 2\varepsilon_r \varepsilon_0 RT \sum_i [M_i]_{bulk} \left(\exp\left\{ - \frac{z_i F \phi_0}{RT} \right\} - 1 \right) \right\}^{1/2} \qquad (6)$$

where $[M_i]_{bulk}$ is the bulk concentration of ions, z_i is their valence, ε_r and ε_0 is the dielectric constant of the diffuse layer and free space, respectively, F is the Faraday constant, R is the gas constant, and T is the absolute temperature.

The surface concentration of phospholipid can be expressed by its volume concentration as follows:

$$[P]_{os} = \frac{V}{S} [P]_0 \qquad (7)$$

where S is the entire surface of phospholipid aggregates and V is the volume of the sample. Using Eqns. 3, 6 and 7, and taking into account that:

$$[Mn^{2+}]_{bulk} = [Mn^{2+}]_f = [Mn^{2+}]_0 - [Mn^{2+}]_b \qquad (8)$$

we obtain a dependence of the surface potential ϕ_0 upon temperature, which can be numerically solved for a given set of parameters $[Mn^{2+}]_0$, $[P]_0$, $(K_a)_{Mn^{2+}}$, $(K_a)_{Cl^-}$, $\beta_{Mn^{2+}}$, β_{Cl^-}:

$$\frac{1}{2\varepsilon_r \varepsilon_0 RT} \left\{ \frac{2e}{s} \cdot \frac{(K_a)_{Mn^{2+}} [Mn^{2+}]_0}{\beta_{Mn^{2+}} + (K_a)_{Mn^{2+}} [P]_0} - \frac{2e}{s} \cdot \frac{(K_a)_{Cl^-} [Mn^{2+}]_0}{\beta_{Cl^-} + (K_a)_{Cl^-} [P]_0} \right\}^2$$

$$= \frac{\beta_{Mn^{2+}} [Mn^{2+}]_0}{\beta_{Mn^{2+}} + (K_a)_{Mn^{2+}} [P]_0} \cdot \left(\exp\left\{ - \frac{2F\phi_0}{RT} \right\} - 1 \right)$$

$$+ \frac{2\beta_{Cl^-} [Mn^{2+}]_0}{\beta_{Cl^-} + (K_a)_{Cl^-} [P]_0} \cdot \left(\exp\left\{ \frac{F\phi_0}{RT} \right\} - 1 \right) \qquad (9)$$

where $(K_a)_{Mn^{2+}}$ and $(K_a)_{Cl^-}$ are apparent association constants of manganous and chloride ions, respectively, $[Mn^{2+}]_0$ is the entire concentration of manganous cations which is equal to the concentration of the used $MnCl_2$ solution, and for the concentration of chloride anions we have written $[Cl^-]_0 = 2[Mn^{2+}]_0$.

From our experiments, we have no data available on the adsorption of chloride anions to phospholipids, because this interaction cannot be detected by ESR spectrometry. According to the results of electrophoretic and ^{31}P-NMR measurements, the values of apparent association constants of chloride anions and phosphatidylcholine lie between 0.025 and 0.065 M^{-1}, being higher in the presence of polyvalent cations and depending on the concentration of polyvalent cations [3,31–35]. In our case, the following surface reactions can be expected:

$$Cl^- + P \rightleftarrows ClP^-$$

$$Cl^- + [Mn(H_2O)_4P_2]^{2+} \rightleftarrows [Mn(H_2O)_4P_2Cl]^+$$

$$Cl^- + [Mn(H_2O)_5P]^{2+} \rightleftarrows [Mn(H_2O)_5PCl]^+$$

These mechanisms would obviously lead to a greater adsorption of chloride anions in the presence of manganous cations. In order to describe such reactions, a further binding constant should be introduced, making the calculations much more complicated. As the association of cations and zwitterionic phospholipids is low, the distribution

of positive charge seen by chloride anions on the surface of phospholipid aggregates is discontinuous, and the influence of discrete charges should be considered. This is rather difficult, because the distribution of charge depends on the concentration of cations, state of phospholipid, temperature, etc. Because of the low value of the association constant of chloride anions, $(K_a)_{Cl^-} = (0.025–0.065)\ M^{-1}$ in comparison to that of manganous cations, $(K_a)_{Mn^{2+}} = (6.4–14.0)\ M^{-1}$, and because of low concentrations of $MnCl_2$ solutions that we have used $[Mn^{2+}]_0 = (10^{-3}–10^{-2})$ M, the adsorption of chloride anions will be neglected in further considerations. Then Eqn. 9 will have the form:

$$\frac{1}{2\varepsilon_r\varepsilon_0 RT}\left(\frac{2e}{s}\cdot\frac{K_a[Mn^{2+}]_0}{\beta + K_a[P]_0}\right)^2 = \frac{\beta[Mn^{2+}]_0}{\beta + K_a[P]_0}\cdot\left(\exp\left\{-\frac{2F\phi_0}{RT}\right\} - 1\right) + 2[Mn^{2+}]_0\cdot\left(\exp\left\{\frac{F\phi_0}{RT}\right\} - 1\right) \qquad (10)$$

where we have written for simplicity $(K_a)_{Mn^{2+}} = K_a$, $\beta_{Mn^{2+}} = \beta$, $\beta_{Cl^-} = 1$.

If we suppose that the surface occupied by one DPPC molecule is $s_1 = 50 \cdot 10^{-20}$ m^2 in the gel phase and $s_2 = 60 \cdot 10^{-20}$ m^2 in the liquid-crystalline phase [23], and if we insert into Eqn. 10 the experimentally acquired values of the apparent association constant $K_{a1} = 14.0\ M^{-1}$ and $K_{a2} = 6.4\ M^{-1}$, we can calculate the surface potential of DPPC aggregates with adsorbed manganous ions $\phi_{01} = 33$ mV and $\phi_{02} = 13$ mV in the gel and liquid-crystalline phase of DPPC, respectively. By calculations we have supposed that the dielectric constant of the diffuse layer is equal to the one of water.

The surface potential in the liquid-crystalline phase of DPPC with adsorbed ions can be estimated also from the Arrhenius plot of K_a (Fig. 4), $\phi_{02} = (9 \pm 5)$ mV. This is in a good agreement with the above calculated value. By extrapolation of the linear Arrhenius plot, we obtained the value of the intrinsic association constant of manganous ions and liquid-crystalline DPPC, $K_i = (13 \pm 5)\ M^{-1}$. In the gel-phase region, the experimentally acquired temperature dependence of K_a was not in accord with Eqn. 2, therefore it was not possible to estimate the surface potential and the intrinsic association constant.

In all calculations, we have used the stoichiometry parameter $\beta = 1$. As stated above, this is relevant to the situation when all phosphate moieties of polar head-groups are accessible to manganous ions and the binding stoichiometry is 1 : 1. On the other hand, if we assume that always two manganous ions bind to one DPPC molecule, the choice of $\beta = 1$ is relevant to the case when half of all phosphate groups is accessible to ions. It must be noticed that the value of the surface potential calculated according to Eqn. 10 is independent of the choice of β.

Cholesterol is known to influence the fluidity of phospholipid systems [36–38]. In order to examine whether it can influence also the electric properties of zwitterionic phospholipids, we have measured apparent association constants of manganous ions and mixtures of DPPC and sphingomyelin with cholesterol (Fig. 5). In calculations of K_a according to Eqn. 4, we have assumed that manganous ions adsorbed only to phospholipids and not to cholesterol. Therefore, we have inserted the total DPPC or sphingomyelin concentration for $[P]_0$. Both temperature dependences of K_a in Fig. 5 exhibit no discontinuity, absolute values of K_a being similar to those of liquid-crystalline DPPC and sphingomyelin (see Fig. 3). Our results are in agreement with results of Inoko et al. [39] who

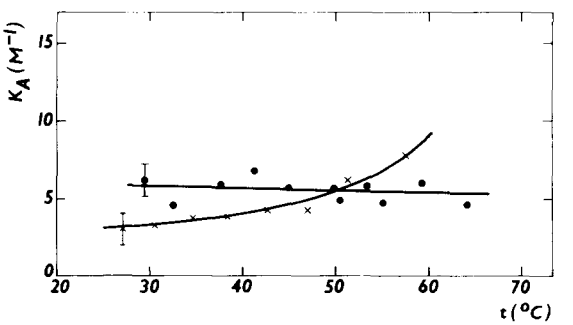

Fig. 5. Temperature dependences of apparent association constants of Mn^{2+}, and mixture of DPPC with cholesterol in mole ratio 1 : 0.37 (●), mixture of sphingomyelin with cholesterol in mole ratio 1 : 0.30 (×), in water dispersions containing 28.0 wt% and 26.5 wt% of the total lipid, respectively. The concentration of $MnCl_2$ was always 5.7 mM.

found by X-ray diffraction measurements that the interaction of some other polyvalent cations with DPPC in the gel phase is smaller in the presence of cholesterol.

From ^{31}P-NMR studies, it is known [40] that cholesterol makes aliphatic chains of phospholipids in the gel phase more fluid, while in the liquid-crystalline phase it lowers their mobility. The conformation of polar heads in both phases is similar to the one of pure liquid-crystalline DPPC, where intermolecular interactions between neighbouring polar heads are lowered or eliminated. Which of these effects of cholesterol on the conformation and association of phospholipid molecules is responsible for the lowering of adsorption of manganous ions to the investigated phospholipids in the gel phase cannot be decided on the basis of our study.

Conclusion

Specific biomembranes have in general a specific composition [41]. A proper lipid composition seems to be necessary for their optimal function. Revealing dependences between the composition and functions of biomembranes is a two-step process. The first step involves gathering of knowledge on behaviour of each lipid as well as revealing exact lipid compositions of specific biomembranes. The second step involves combining this information with information on functions of specific membranes. The aim of our work was to contribute to the first step. The following general conclusions can be drawn from our results:

(1) The adsorption of manganous ions to zwitterionic phospholipids with phosphorylcholine headgroups is greater in the gel phase than in the liquid-crystalline phase.

(2) The temperature dependence of adsorption to liquid-crystalline phospholipids is exponential. In the gel phase, a change of adsorption can be expected at the pretransition of the lipid.

(3) Cholesterol decreases the affinity of manganous ions to DPPC and sphingomyelin in the gel phase.

(4) The adsorption of manganous ions to pure DPPE is much lower than to DPPC or sphingomyelin, with no change at the main phase transition.

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