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DIVALENT CATION BINDING TO PHOSPHOLIPID VESICLES

DEPENDENCE ON TEMPERATURE AND LIPID FLUIDITY

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Summary

Mn^{2+} binding to vesicles prepared from several different species of anionic phospholipids was determined as a function of temperature by electron paramagnetic resonance (EPR). The Mn^{2+} affinities of phosphatidylserine, cardiolipin and egg yolk phosphatidylglycerol all increased monitonically with temperature.

Vesicles prepared from hydrogenated and natural (bovine) phosphatidylserine were monitored with respect to hydrocarbon chain fluidity as well as Mn^{2+} binding. Contrary to expectations based on surface potential considerations, the affinity of phosphatidylserine for divalent cations was apparently not lowered in going from the gel state to the liquid crystalline state of the bilayer. The results are instead consistent with an enhancement in cation affinity with increased lipid fluidity.

Dipalmitoyl phosphatidylglycerol vesicle fluidity and Mn^{2+} binding were also studied with EPR. A large reduction in the measured Mn^{2+} affinity accompanied melting of the phospholipid, but observed hysteresis in the temperature dependence of the binding render uncertain any simple explanation based on changes in surface potential. Supplementary light scattering data indicated that vesicle aggregation was involved in the hysteresis phenomena.

Introduction

The binding of divalent cations tends to condense anionic phospholipid membranes, reducing the mean separation between polar head groups and the

Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; DPPG, dipalmitoyl phosphatidylglycerol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

fluidity of the hydrocarbon chains [1—4]. It is likely, moreover, that immobilization of the fatty acyl chains of acidic phospholipids plays a role in divalent cation-induced phase separations [4,5] in mixed bilayers containing phosphatidylcholine.

The molecular mechanism of divalent cation binding to phosphatidylserine and other charged phospholipids is poorly understood, but it has been suggested that the bound cation may bridge head groups of neighboring phospholipids [1,6,7]. Whether or not such complexes are formed, divalent cation binding to the head groups will be strongly influenced by the close packing of lipid molecules into a bilayer structure.

First, the mobile divalent cation concentration in the aqueous phase very close to an anionic surface is elevated over its value in the bulk solution because of the electrical field in the interface region. There will be, as a consequence, a corresponding enhancement in the apparent affinity of surface groups for binding the cation. The magnitude of this effect can be estimated from Gouy-Chapman and Stern equations, as reviewed by McLaughlin [8]. Thus, the apparent affinity, K_A, of a divalent cation for a charged membrane is, according to the Stern approximation:

$$K_A = K_0(-2e\psi_0/kT) \tag{1}$$

where K_0 is the binding affinity uncorrected for the electrostatic effect, ψ_0 is the surface potential, and the other symbols have their usual meanings.

We shall be interested in cases where the concentrations of divalent cations is small enough compared to the concentrations of monovalent cations and charged phospholipids that the influence of divalent cations on the surface potential can be neglected (see ref. 7). If it is further assumed that, in contrast to the divalent cations, monovalent ions only 'screen' but do not 'bind' to the surface [7,9], the usual expression for the surface potential follows from the Gouy-Chapman theory:

$$\sinh e\psi_0/2kT = \sigma/(8NC\epsilon\epsilon_0 kT)^{1/2} \tag{2}$$

where N is Avogodro's number, ϵ is the dielectric constant of water, C is the monovalent salt concentration, and σ is the surface charge density. For large negative σ and moderate C, Eqns. 1 and 2 can be combined to obtain:

$$K_A = K_0\sigma^4/(2NC\epsilon\epsilon_0 kT)^2 \tag{3}$$

Since the mean separation between phospholipid head groups generally increases with hydrocarbon chain mobility, it follows from Eqn. 3 that, neglecting possible shifts in K_0, K_A should decrease as the bilayer is made more fluid, e.g. by raising temperature.

Furthermore, the immobilization of hydrocarbon chains concomitant with divalent cation adsorption to phospholipid bilayers indicates that the free energies of the chains are to some degree dependent on divalent cation association with the head groups. Since the equilibrium constant for any reaction, including ion binding, is governed by the total free energy change in the system, the affinity of phospholipids for divalent cations may be modulated in this manner also by the interactions between fatty acid chains.

In this paper the effects of temperature and hydrocarbon chain fluidity on the interactions between anionic phospholipid vesicles and the paramagnetic Ca^{2+} analog Mn^{2+} are critically examined. Evidence in favor of divalent cation bridges between different bilayers under some circumstances is also presented.

Materials and Methods

Materials. Dipalmitoyl phosphatidylglycerol (DPPG) or egg yolk phosphatidylglycerol was prepared enzymatically, essentially as described by Papahadjopoulos et al. [10], from glycerol and L-α-dipalmitoyl phosphatidylcholine (Sigma) or egg yolk phosphatidylcholine (GIBCO), respectively, with the aid of a phospholipase D extract from cabbage (Sigma). Reaction products were eluted from a silicic acid column by a step-wise gradient of chloroform/methanol. Fractions were collected and analyzed by thin-layer chromatography (TLC); those fractions containing phosphatidylglycerol, but no phosphatidylcholine or phosphatidic acid, were kept and pooled for future experiments.

Other phospholipids were obtained commercially and determined to be pure by TLC. The suppliers of these compounds were: bovine phosphatidylserine, GIBCO; hydrogenated bovine phosphatidylserine, P-L Biochemicals; cardiolipin, Sigma.

The spin probes (SYVA) employed in this study were methyl esters of stearic acid having the paramagnetic doxyl group covalently attached at a known position along the chain.

Preparation of vesicle suspensions. Phospholipid vesicles were prepared as described previously [7] except that: (a) for DPPG and hydrogenated phosphatidylserine, sonication was carried out at 55—60° C rather than at room temperature, and (b) Mn^{2+} was routinely added to samples after sonication, just before EPR measurements were to begin. Hence Mn^{2+} was present, at least initially, only on the outside of the vesicles. For fluidity studies, the nitroxide spin probe was dried down together with the appropriate phospholipid from chloroform prior to suspension and sonication of the lipid [11].

EPR measurements. EPR spectra were recorded with a Varian E-9 spectrometer, equipped with dual cavity and variable temperature accessory. Temperature was monitored with a digital voltmeter in conjunction with a thermocouple taped to the aqueous sample cell just above the active region of the cavity. Accuracy in temperature readings was about ±0.3° C. To minimize errors stemming from variations in tuning, each signal amplitude recorded on a vesicle sample was normalized against one recorded immediately afterwards on a pitch standard located in the other dual cavity compartment.

Determinations of vesicle affinities for Mn^{2+}. Mn^{2+} binding to the vesicles was quantitated based on EPR estimates of the free Mn^{2+} fraction [7,12,13]. In order to determine the free manganese concentration in a sample containing phospholipid vesicles, the amplitude of the narrow hyperfine sextet component of the EPR spectrum was compared against that detected at the same temperature with a standard Mn^{2+} solution containing no phospholipid. (Because of the strong dependence on temperature of $Mn(H_2O)_6^{2+}$ spectra [14,15], it was crucial to monitor the standard solution over the same temperature range as the sample.)

Suspensions high in concentrations of phosphatidylserine or cardiolipin but relatively low in Mn^{2+} and monovalent salt were also studied as a function of temperature to gain estimates of possible distortion in the free Mn^{2+} signals by the underlying bound signals [7]. It was found that the bound spectra described earlier [7] varied only slightly in shape over the temperature range of interest. Based on these spectra and those obtained from the free Mn^{2+} standards, corrections were made for the estimated distortion in calculating the affinities of these two lipids for Mn^{2+}; these corrections never exceeded 15%. No correction for the bound signal was applied in calculating Mn^{2+} affinities for the phosphatidylglycerols. Nevertheless, it was deduced from close examination of the recorded sextet spectra that the contribution from broad underlying spectra components was always relatively small.

The molar ratio of Mn^{2+} to phospholipid in all samples was $<<1$. The advantages of this restriction have been discussed previously [7]. Under these conditions the apparent affinity (association constant) K_A can be approximated by the expression:

$$K_A = \frac{\beta[Mn^{2+}]_b}{[Mn^{2+}]_f[P]} \tag{4}$$

where $[Mn^{2+}]_b/[Mn^{2+}]_f$ is the ratio of bound to free manganese, as determined by EPR, [P] is the sample concentration of phospholipid head groups, and β is a normalization factor dependent on the binding stoichiometry and the fraction of head groups accessible to the Mn^{2+} (which in turn is dependent on the size distribution of the vesicles). [P] was calculated from the weight of phospholipid in the sample under the approximation that, for each lipid but DPPG, all fatty acyl chains were 18 carbons long. In those cases which have been investigated, NMR has shown that roughly 2/3 of the head group phosphates in sonicated phospholipid vesicles are exposed to the external solution [16—20]. Based then on an assumed 1 : 1 Mn^{2+}-phospholipid binding stoichiometry, β was set equal to 1.50. Although this remains, to an extent, an arbitrary convention, the conclusions reached here primarily involve comparative values of K_A and are therefore essentially independent of the magnitude of β. (β could also include a correction for the intravesicular space which is inaccessible to Mn^{2+}, but based on published estimates of trapped volume in phospholipid dispersions [21,22], this correction is expected to be negligible.)

Light scattering measurements. Light scattering by DPPG suspensions was monitored with a fully-corrected Aminco-Bowman spectrofluorimeter, equipped with a temperature-regulating water jacket surrounding the cuvette. The excitation and emission monochrometers were adjusted to the same wavelength (520 nm) so as to detect Raleigh scattering. The machine was operated in the 'RATIO' mode to correct for instrumental drifts.

Ordinarily, 15 min were allowed between successive readings at different temperatures. Temperature was sensed with a Markson probe briefly inserted into the cuvette 2 min before a light scattering measurement was to be taken. The sample was continually mixed with a small magnetic stir bar placed in the cuvette except for short periods during which the light scattering was monitored.

Results

Temperature dependence of Mn^{2+} binding to unsaturated phospholipids

When Mn^{2+} was added to the outside of phosphatidylserine vesicles and binding followed with EPR, the apparent affinity increased monitonically as the temperature was raised from 15 to 65°C (Fig. 1, open triangles). K_A was, as described in Materials and Methods, calculated on the basis of one site per external head group; hence, exposure of more binding sites can cause an artifactual increase in K_A. It is therefore significant that when the sample was cooled back to 15°C, the calculated K_A was only slightly higher than it was before heating (see solid triangle); thus, Mn^{2+} penetration into the vesicles was not responsible for more than a small fraction of the observed increase in binding with temperature.

As illustrated in Fig. 2, Mn^{2+} binding to cardiolipin and egg yolk phosphatidylglycerol vesicles also became stronger with increasing temperature. Mn^{2+} permeability was relatively greater in these samples than with phosphatidylserine: for this reason, the measurements were not extended above 55°C.

Data like that in Figs. 1 and 2, when replotted as van't Hoff ($\log K_A$ vs. $1/T$) graphs, can be used to derive estimates of the standard enthalpy changes for the binding reactions. The values of ΔH (in kcal/mol) arrived at in this way from least squares fits to several sets of data on each lipid were as follows: bovine phosphatidylserine, 4.17 ± 0.19; hydrogenated phosphatidylserine, 3.52 ± 0.21; cardiolipin, 4.58 ± 0.41; egg yolk phosphatidylglycerol, 2.51 ± 0.34. It should be noted that only heating runs were used to generate the van't Hoff plots; consequently Mn^{2+} influx may have caused some overestimate in ΔH^0, particularly for egg yolk phosphatidylglycerol and cardiolipin. Nevertheless, the sign of ΔH^0 was clearly positive for these lipids as well as for phosphatidylserine (see Fig. 2).

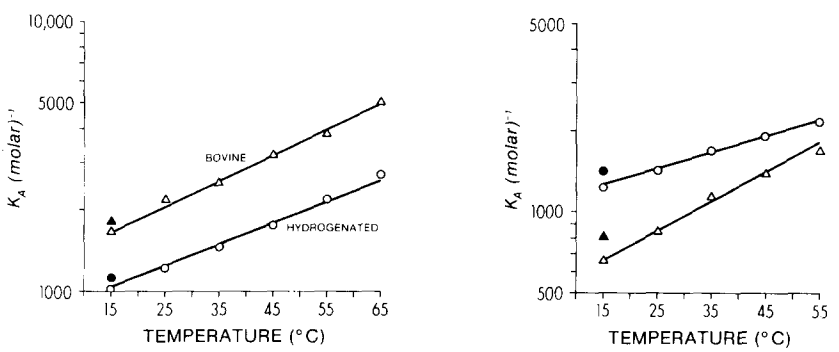

Fig. 1. Mn^{2+} binding to phosphatidylserine. Samples contained 160 mM NaCl, 20 mM Na-HEPES (pH 7.3), 0.14 mM $MnCl_2$ and 4.3 mg/ml of bovine (△) or hydrogenated (○) phosphatidylserine. The respective closed symbols denote the affinity measured after temperature recycling.

Fig. 2. Mn^{2+} binding to egg yolk phosphatidylglycerol and cardiolipin. The egg yolk phosphatidylglycerol sample (○) contained 3 mg/ml phospholipid, 80 mM NaCl, 15 mM Na-HEPES (pH 7.3) and 0.1 mM $MnCl_2$. the cardiolipin vesicle suspension (△) contained 6 mg/ml lipid, 20 mM Na-HEPES (pH 7.3), 100 mM NaCl and 0.14 mM $MnCl_2$. Again, the closed symbols show the increase in binding after temperature recycling.

Positive enthalpy changes of roughly the magnitudes cited above have been reported for the formation of some aqueous Mn^{2+} complexes [13,23]. Thus the observed increases in K_A with temperature may simply reflect an enhanced affinity of the individual head group moieties for Mn^{2+}. Alternatively, a positive ΔH^0 may arise from a 'membrane effect' involving, e.g. the reduction in hydrocarbon chain mobility which accompanies divalent cation complexation. In other words, the increases in K_A with heating may be related to the enhancement of fluidity with rising temperature. If so, alterations in the fatty acid composition of a phospholipid might change its affinity for cations.

Comparison of saturated and unsaturated phosphatidylserine

To explore the relationship between phospholipid fluidity and divalent cation affinity, vesicles respectively composed of natural bovine phosphatidylserine and of phosphatidylserine which had its fatty acyl chains catalytically hydrogenated were compared with respect to both fluidity and Mn^{2+} binding.

Comparison of fluidity. First, the spin probe 16-doxylstearate (methylester) was incorporated into the two types of phosphatidylserine vesicles. EPR spectra were monitored as a function of temperature, and the fluidity was characterized by the quantity τ_c defined by the expression [24,25]:

$$\tau_c = 6.5 \cdot 10^{-10} W_2 [(h_2/h_3)^{1/2} - 1] \text{ s} \quad (5)$$

where h_2/h_3 is the ratio of the peak-to-peak heights of the middle and high field lines and where W_2 is the peak-to-peak widths of the middle line (in Gauss). When the probe motion is roughly isotropic and $\tau_c < 10^{-9}$ s, τ_c provides a good estimate of the rotational correlation time [24,25], otherwise it should be regarded simply as a parameter which is sensitive to changes in probe mobility. At a given temperature τ_c was, as can be seen in Fig. 3, larger in vesicles of the hydrogenated species indicating, as expected, a more rigid environment for the probe.

Interestingly, the curves obtained from the two kinds of vesicles were nearly parallel so that probe mobility at a given temperature in saturated phosphatidylserine appeared to be about the same as that in unsaturated phosphatidylserine at approx. 26°C lower temperature. Differential scanning calorimetry measurements have indicated that bovine phosphatidylserine undergoes a rather broad phase transition near 5°C [4]. Thus, while there was no detectable phase transition for either lipid in Fig. 3, the data suggest that the hydrogenated phosphatidylserine may melt at ≈31°C. (The absence of a discontinuity in Fig. 3 may be attributable to the gradual nature of the transition or perhaps to the choice of spin label.)

Phosphatidylserine vesicles doped with the 5-doxyl- and 12-doxyl-stearate (methyl ester) spin probes were also examined with EPR. The hyperfine splitting component $2A'_{\prime\prime}$ was determined as a function of temperature according to the method of Hubbell and McConnell [26] for randomly oriented samples. As discussed by the same authors [26], the magnitude of $2A'_{\prime\prime}$ generally increases as the hydrocarbon chains become more extended.

The results obtained from these probes located in hydrogenated phosphatidylserine vesicles, as shown by the circles in Fig. 4, thus seem to indicate a transition from an ordered to disordered state, extending perhaps over the

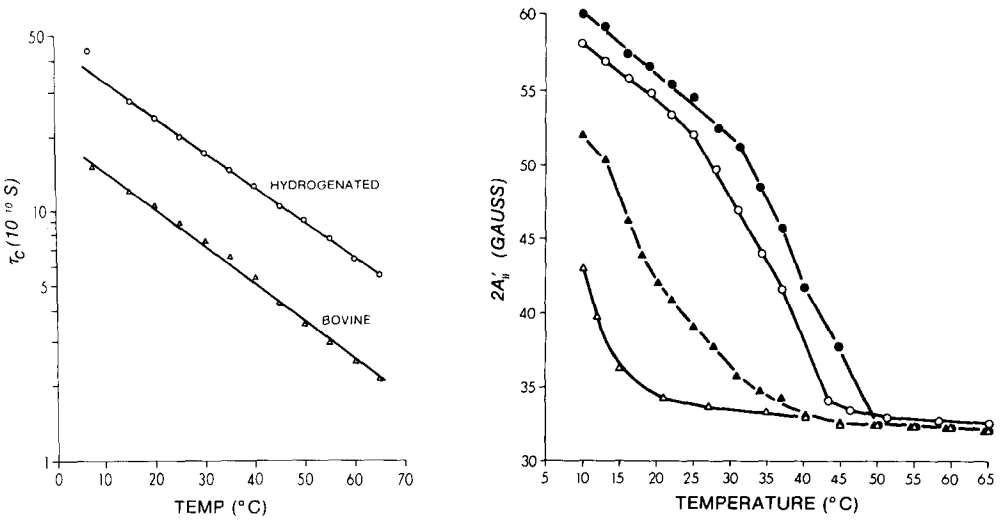

Fig. 3. Fluidity of hydrogenated and bovine phosphatidylserine vesicles. Samples 8 mg/ml of phospholipid, 0.05 mg/ml of the 16-doxyl-stearate (methyl ester) spin probe, 160 mM NaCl, and 20 mM HEPES (pH 7.3).

Fig. 4. EPR study of phosphatidylserine vesicle fluidity. Suspensions contained 10 mg/ml of either hydrogenated (circles) or bovine (triangles) phosphatidylserine cosonicated with 0.12 mg/ml of either 5-doxyl- (closed symbols) or 12-doxyl- (open symbols) stearate (methyl ester) spin label.

range from ≈30 to 50°C. However, when those results are carefully compared with data derived from similarly labeled bovine phosphatidylserine vesicles (Fig. 4, triangles) the interpretation of the decreases in $2A'_{II}$ with heating become less certain, especially in light of the calorimetry findings cited above [4]. The results in Fig. 4 are, nevertheless, at least in qualitative agreement with those in Fig. 3, i.e. they show that the fluidity of hydrogenated phosphatidylserine corresponds to that of bovine phosphatidylserine at roughly 25°C lower temperature. Hence, taken in conjunction with the previous calorimetry data, they again indicate that hydrogenated phosphatidylserine melts near 30°C.

Comparison of Mn^{2+} binding. In planar phospholipid bilayers, the area/molecule increases by ≈50% in going from the gel to liquid crystalline phase [27,28]. Recently, Watts et al. [29] have reported that the area/molecule in the outer leaflet of dimiristoyl phosphatidylcholine vesicles likewise increases during melting from 45.3 ± 3.6 to 74.7 ± 3.7 Å^2. If these findings can be extrapolated to vesicles composed of charged phospholipids, it follows that the magnitude of the surface charge density σ on the outside of such vesicles would be 50—80% higher in the gel state than in the liquid crystalline state. Then from the standpoint of surface potential alone, the Gouy-Chapman and Stern approximations (Eqn. 3) predict, as a consequence, a 5—10-fold enhancement of divalent cation affinity for vesicles in the gel state.

In light of the spin label data presented above, one might then expect to a first approximation, that: (a) below ≈30°C, vesicles of the hydrogenated phosphatidylserine should bind Mn^{2+} substantially (approx. 5—10 times) more tightly than those made from natural bovine phosphatidylserine and (b) the affinity of the hydrogenated species for Mn^{2+} should (perhaps gradually) fall

off by roughly a factor of 5 or 10 as the vesicles are heated through the posited transition beginning near 30°C.

The affinity of hydrogenated phosphatidylserine for Mn^{2+} was determined under the same set of conditions employed with unsaturated bovine phosphatidylserine. For comparison purposes, both sets of results are plotted together in Fig. 1. Clearly, neither prediction put forth in the preceding paragraph was fulfilled. Binding to hydrogenated phosphatidylserine increased almost linearly as the temperature was raised from 15 to 65°C, the apparent enthalpy change being slightly lower than for the unsaturated phosphatidylserine (see Fig. 1). The binding affinity of the former was, moreover, actually weaker than that of the natural lipid over the entire temperature range. Therefore, while these results suggest that divalent cation binding to anionic phospholipids may be dependent, to some degree, on hydrocarbon chain fluidity, they also appear, at first sight, to conflict strongly with the theoretical approach outlined in Introduction. These points will be further addressed in Discussion.

Studies on DPPG vesicles

The fluidity and Mn^{2+} binding properties of enzymatically prepared DPPG were also investigated with EPR. DPPG which had been sonicated together with the spin label 16-doxyl-stearate (methyl ester) at 55°C and then cooled displayed a definite phase transition at $T_c \approx 40°C$ during subsequent heating (Fig. 5). As also shown in Fig. 5, the fluidity curve was, with minor modifications, reversible upon cooling. A phase transition in the range 32—40°C was also seen in plots of spectral parameters derived from EPR measurements with the methyl esters of 5- and 12-doxyl-stearate (data not shown). The location of the transition, as reported by the spin labels, is in good agreement with values obtained by differential scanning calorimetry and fluorescence [4,9].

As discussed previously, the Gouy-Chapman theory predicts that from the point of view of surface potential and surface charge density alone (Eqn. 3), the affinity of DPPG for divalent cations should be 5—10 times higher when $T < T_c$ than when $T > T_c$. As shown in Fig. 6a there was in fact a fairly abrupt increase in binding when vesicles treated with Mn^{2+} at 60°C were cooled below the phase transition. Upon reheating the sample to 60°C, the affinity dropped again to near its initial value, but the reheating curve (Fig. 6b) appeared displaced with respect to the cooling curve (Fig. 6a); this hysteresis is puzzling in light of the reversible behavior found in the spin label data of Fig. 5.

More significantly, when Mn^{2+} was added to a suspension which had been sonicated at 60°C but precooled to 20°C, the measured K_A (Fig. 7a) was much lower than that found after the vesicles were cooled to 20°C in the presence of Mn^{2+} (Fig. 6a). As further illustrated in Fig. 7a, heating caused a rise in binding which peaked near the phase transition, the affinity falling off once again at higher temperatures. When the temperature was again lowered from 60 to 20°C (Fig. 7b) the affinity again rose sharply near the phase transition and then plateaued as it did in the initial cooling run of Fig. 6a. Hence the affinity at 20°C was enhanced dramatically simply by heating and cooling the sample. Since the data of Fig. 5 clearly indicate that the vesicles are in the gel state at 20°C, in the presence or absence of Mn^{2+}, irrespective of prior exposure to

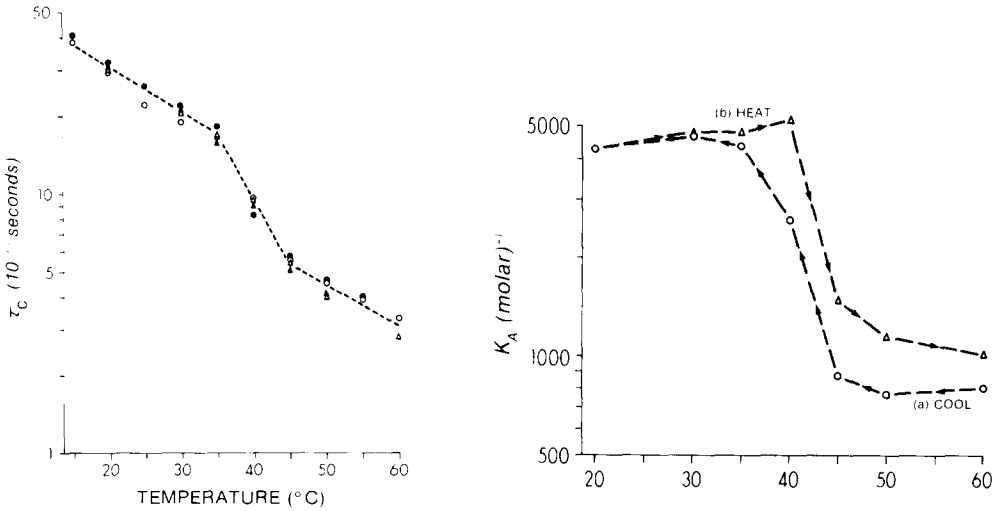

Fig. 5. Spin label study of DPPG vesicle fluidity. Samples contained: 3 mg/ml DPPG, 0.02 mg/ml 16-doxyl-stearate (methyl ester), 15 mM HEPES (pH 7.3) and 250 mM NaCl. Vesicles were rapidly cooled to 0°C following sonication at 55°C. In the case of the triangles, 0.14 mM Mn^{2+} was added in a small volume to the cooled suspension. EPR spectra were recorded first as a function of increasing (open symbols) and then decreasing (closed symbols) temperature.

Fig. 6. Mn^{2+} binding to DPPG. $MnCl_2$ (0.14 mM) was added to a DPPG vesicle suspension at 60°C just after sonication. The sample was kept at approximately this temperature as it was transferred to the spectrometer, and binding was monitored as a function of (a) decreasing (○) and then (b) increasing (△) temperature. (Approx. 10 min elapsed between measurements at successive temperatures.) The samples contained, in addition to Mn^{2+} and 3 mg/ml DPPG, 250 mM NaCl and 15 mM HEPES buffer (pH 7.3).

higher temperatures, the change in binding at 20°C cannot be attributed solely to a change in surface charge density associated with altered packing of the hydrocarbon chains. Specifically, supercooling phenomena of the type reported by Ververgaert et al. [30] cannot account for this effect.

Other nonreversible changes and hysteresis effects have been noted during the heating and cooling of phospholipid vesicles, especially in passing through a phase (or pre-phase) transition [31—33]. Apparently, vesicle-vesicle aggregation is often involved in these phenomena [31—33]. To investigate the possible relationship between divalent cation binding and vesicle aggregation, light scattering by a sonicated dispersion of DPPG was monitored over a heating/cooling cycle under conditions similar to those employed in the Mn^{2+} binding experiment of Fig. 7. As shown in Fig. 8a, a modest increase in scattering was observed while warming to approximately T_c, followed by a plateau and a fall-off near 60°C. Recooling the sample however, induced large increases in light scattering (Fig. 8b) indicating substantial vesicle aggregation. When EDTA was added to chelate the Mn^{2+} after completion of the cooling run (Fig. 8c), the light scattering slowly decreased over a 10 min time interval, finally reaching a steady-state intermediate between that observed at the beginning and end of the heating/cooling cycle. The vesicles then appeared to disaggregate further as the suspension was again heated through the phase transition (Fig. 8d).

These results indicate a close connection between vesicle aggregation and the hysteresis in Mn^{2+} binding. In particular, binding was enhanced along with

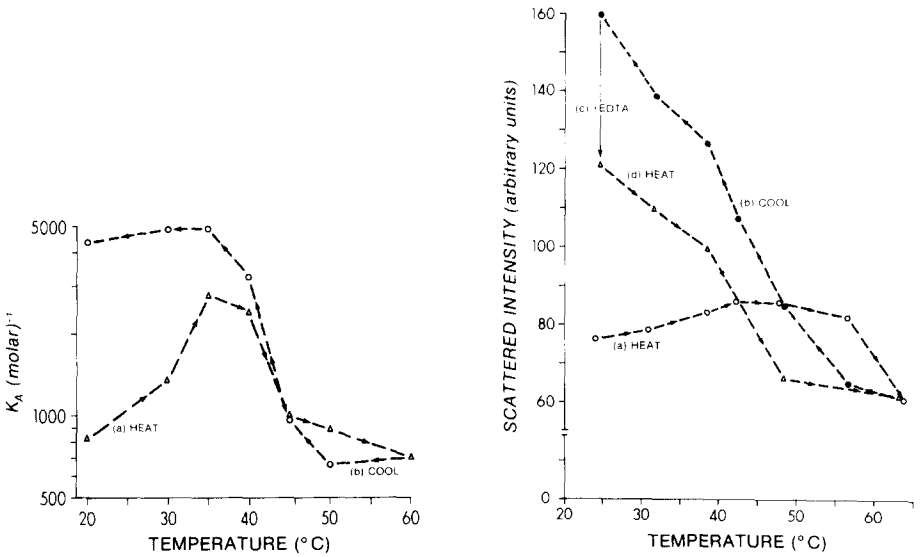

Fig. 7. Mn^{2+} binding to DPPG. Conditions were as in Fig. 6 except that the suspension was cooled to room temprature prior to Mn^{2+} addition. Binding was then determined as a function of first (a) increasing (△) and then (b) decreasing (○) temperature.

Fig. 8. Light scattering by DPPG suspension. DPPG (2.4 mg) was suspended and sonicated at ≈60°C in a 1 ml volume of 250 mM NaCl, 20 mM HEPES (pH 7.3). The suspension was rapidly cooled and transferred to a cuvette in the fluorimeter at 23.7°C. After the light scattering at 520 nm was noted, $MnCl_2$ (0.1 mM) was added in a small volume with no appreciable perturbation in the measured light scattering. The scattered intensity was then monitored sequentially as a function of (a) increasing and then (b) decreasing temperature. At the conclusion of the cooling cycle, 10 μl of a 200 mM EDTA solution was added. As indicated by the arrow (c), the observed scattering decreased slow, reaching a new steady state after a few minutes. Finally, the scattered intensity was monitored as a function of increasing temperature (d).

aggregation as the vesicles were slowly cooled through the phase transition. At the same time, it appears that the divalent cations helped to promote aggregation since EDTA caused a decrease in light scattering. Possible mechanisms for explaining this behavior are treated in Discussion.

Discussion

Mn^{2+} affinities for the exterior of phosphatidylserine, egg yolk phosphatidylglycerol all increased monitonically with temperature. This may, as mentioned earlier, simply reflect the nature of cation complexation by the isolated head group moieties. However, changes in hydrocarbon chain fluidity may be more important. In particular, complexation of divalent cations by the phospholipid head groups is expected to make the hydrocarbon chains more rigid and extended [1—4]. Associated with this transformation will be a reduction in the enthalpy and entropy of the hydrocarbon phase and a resultant free energy change ΔG^0_{HC} which, in principle, may be either $>$ or <0. Since the Mn^{2+} affinity is a function of the standard free energy change in the whole system, hydrocarbon chain ridigification will, through ΔG^0_{HC}, influence the magnitude of K_A and its dependence on temperature. Particularly significant in this regard

may be variations in ΔG^0_{HC} itself with temperature as a result of altered hydrocarbon chain fluidity.

That hydrogenated phosphatidylserine bound Mn^{2+} less tightly than unsaturated phosphatidylserine supports the hypothesis that hydrocarbon chain immobilization may help to stabilize head group complexation of divalent cations. It is interesting in this connection, moreover, that the former exhibited roughly the same affinity as the latter did at $\approx 25°C$ lower temperature (Fig. 1). In view of the spin label data (Figs. 3 and 4) which showed a similar correspondence, this result further suggests that hydrocarbon chain fluidity is an important factor in Mn^{2+}-phospholipid binding.

The spin label results of Figs. 3 and 4 suggested a broad phase transition beginning at $\approx 30°C$ for the hydrogenated phosphatidylserine vesicles. Although the Gouy-Chapman and Stern equations predict a strong enhancement of Mn^{2+} affinity below this point, it was not observed (Fig. 1). This discrepancy could reflect a serious breakdown in the theory, but other explanations are possible. First, the very tight packing of polar head groups expected in the gel state may not be present in these vesicles below 30°C despite the strong ordering indicated by the spin probes. Alternatively, the phase transition of hydrogenated phosphatidylserine might extend to much higher temperatures than suggested by the data in Fig. 4, the probes preferentially partitioning into more fluid regions. In this case, the vesicles might remain predominantly in the gel state over the entire temperature range 15—65°C in Fig. 1 so that no appreciable shifts in surface charge density occur during the scan. This would not, however, help to account for the tighter Mn^{2+} binding to the unsaturated phosphatidylserine which is expected to be in the liquid crystalline phase over the entire range.

Still another interpretation of these results might be predicated on the assumption that complexation of Mn^{2+} by phosphatidylserine may involve displacement of Na^+ or H^+ from the surface of the vesicles [34]. Thus, if the binding reaction were actually given by:

$$Mn^{2+} + 2(Na\text{-}PS) \rightarrow Mn\text{-}PS_2 + 2\ Na^+$$

or

$$Mn^{2+} + 2\ PS^- \rightarrow (Mn\text{-}PS_2)^{2-} + 2\ H^+\ ,$$

the affinity, according to the theory, would no longer depend on surface potential or surface charge density. (It should be noted, however, that the mechanism involving ejection of two protons appears in conflict with the observed steep dependence of K_A on monovalent salt concentration [7].)

DPPG binding of Mn^{2+}, on the other hand, increased sharply as vesicles were cooled through the phase transition near 40°C (Figs. 6a and 7b). Nevertheless the relatively low K_A observed at 20°C prior to any temperature cycling (Fig. 7a) and the hysteresis shown in Figs. 6 and 7 during heating and cooling cast some doubt on a simple explanation based on a change in surface charge density. Parallel light scattering measurements, moreover, indicated a complex vesicle aggregation behavior, which to some degree paralleled the nonreversible characteristics of Mn^{2+} binding shown by EPR. In particular, both binding and aggregation at temperatures well below T_c were greatly enhanced by cycling

the sample through the phase transition.

One possible interpretation of the data is that Mn^{2+} binding is stabilized by aggregation of the vesicles. This might occur if Mn^{2+} forms tight bridges between closely opposed vesicle surfaces. Supporting this view was the observation that EDTA partially reversed the extensive aggregation observed at low temperatures in the presence of Mn^{2+}. (Divalent cation bridging between acidic phospholipid vesicles has been previously suggested by Portis and Papahadjopoulos [35].)

Alternatively, from Eqn. 4 and the discussion immediately following, it is clear that the hysteresis in the calculated K_A may, in principle, reflect changes in the number of accessible sites as well as in their affinities. For example, it might be hypothesized that, below T_c, the vesicles are aggregated so as to shield many of the sites on the exterior of the vesicles. Since Mn^{2+} added at 20°C can only bind to the small fraction of sites which are exposed, the K_A appears to be low. With heating, Mn^{2+} penetration to the shielded sites may be enhanced so the binding increases (see Fig. 7a). Heating above T_c causes disaggregation, but also decreased binding (Fig. 7a) as the change in surface charge density and surface potential brings about an actual reduction in the affinity of available sites in accordance with theoretical prediction. (Hence this interpretation is qualitatively consistent with the Gouy-Chapman theory.) Finally, as the sample is recooled below T_c (Fig. 7b), Mn^{2+} is trapped between the vesicles as they reaggregate. In this case, both the number and affinity of available sites are high so the observed binding is also high.

Changes in the number of accessible sites would also occur if the vesicles became permeable. The hysteresis seen in Figs. 7 and 8 might thus alternatively (or additionally) be related to enhanced vesicle permeability near the phase transition temperature [10]. To account for the large changes in binding at 20°C upon recycling (Fig. 7) by this mechanism alone would, however, also require that DPPG molecules in the inner leaflet bind Mn^{2+} much more strongly than those exposed externally when the bilayer is in the gel state.

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