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INTERACTIONS OF La^{3+} WITH PHOSPHATIDYLSERINE VESICLES

BINDING, PHASE TRANSITION, LEAKAGE, ^{31}P-NMR AND FUSION

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The interaction of La^{3+} with phosphatidylserine vesicles is studied by differential scanning calorimetry, ^{140}La binding, ^{31}P-NMR chemical shifts and relaxation rates, carboxyfluorescein and [^{14}C]sucrose release, X-ray diffraction and freeze-fracture electron microscopy. In the presence of La^{3+} concentrations above 1 mM and an incubation temperature of 38°C, i.e., at the phase transition temperature of the complex La/phosphatidylserine, the binding ratio of La/lipid exceeds a 1/3 ratio, reaching saturation at a 1/2 ratio. Analysis, employing a modified Gouy-Chapman equation, indicates a significant increase in the intrinsic binding constant of La/phosphatidylserine when the La^{3+} concentration exceeds the threshold concentration for leakage. The analysis illustrates that at the molecular level the binding of La^{3+} can be comparable to or even weaker than that of Ca^{2+}, but that even when present at smaller concentrations La^{3+} competes with and partially displaces Ca^{2+} from membranes or other negatively charged surfaces. The results suggest that the sequence $La^{3+} > Ca^{2+} > Mg^{2+}$ reflects both the binding strength of these cations to phosphatidylserine as well as their ability to induce leakage, enhancement of ^{31}P spin-lattice relaxation rates, fusion and other structural changes. The leakage, fusion, and other structural changes are more pronounced at the phase transition temperature of the La/lipid complex.

Introduction

Previous studies on phosphatidylserine (PS) vesicles demonstrated that Ca^{2+} and Mg^{2+} induced structural changes which have been interpreted to occur by fusion of these vesicles [1–4]. Later, a more direct procedure has elucidated [5] that during the first seconds to minutes the Ca^{2+}-induced structural changes of PS vesicles occur via fusion. It has also been shown that initially the degree of leakage of trapped material per fusion event is small and can be manipulated by varying cation concentrations [6,7]. The studies [7] indicated that under the above conditions (e.g. room temperature and 1–2 mM Ca^{2+}) the rate limiting step of the Ca^{2+}-induced fusion of PS vesicles is the close approach of the vesicles rather than the process of fusion per se.

A preliminary report [8] has shown that La^{3+} produces structural changes of sonicated PS vesicles at threshold concentrations of about 0.1 mM, i.e., 10 and 50 times lower than those required with Ca^{2+} or Mg^{2+}, respectively. The detailed study presented here deals with the interaction of La^{3+} with PS vesicles by employing several experimental

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procedures, and correlates binding capacity of cations (in the sequence Na^+, Mg^{2+}, Ca^{2+}, and La^{3+}) with their effectiveness in causing aggregation and structural changes of these vesicles.

The results of the present study elucidate both experimentally and theoretically the simultaneous effects of several cations (e.g. $La^{3+} + Na^+$ and $La^{3+} + Ca^{2+} + Na^+$) on membrane structures; a subject which has recently been investigated in studies on cellular systems [9–11], and in phosphatidylcholine vesicles [12–17].

Materials and Methods

Leakage of radioactively labeled sucrose

The changes in permeability of liposomes after exposure to La^{3+} were determined as follows.

Liposome preparation. Pure bovine brain phosphatidylserine (PS, >99% (Avanti, Birmingham)) was checked for purity by TLC before use. Sonicated PS liposomes were made as described previously [25,26] in 100 mM NaCl, 4 mM histidine-Tris and 1 mM [^{14}C]sucrose (50 μC/mmol sucrose)/10 μmol PS/ml, pH 7.4. These liposomes were essentially all unilamellar and had a mean diameter of approx. 30 nm [26]. The liposomes were diluted to 4.1 μmol PS/ml with the above buffer and were dialysed three times for 1 h against 100 ml buffer/ml liposomes, and one time for 16 h against 500 ml buffer/ml liposomes to remove non-entrapped sucrose. Sucrose is entrapped in these liposomes in the internal aqueous space.

Permeability measurements. The measurements were carried out essentially using the method described in Ref. 25. Tubes were prepared containing 10 ml of the above buffer plus varying amounts of $LaCl_3$ to give a final $LaCl_3$ concentration between 0.1 and 0.3 mM. In some experiments NaCl concentration was also varied. These tubes were incubated at temperatures ranging from 4 to 60°C. Prewashed 1-cm dialysis bags were prepared as follows: 0.1 ml of the liposomes were placed in the bags which were then placed in the tubes with one end still open. 0.9 ml of NaCl-histidine buffer containing varying concentrations of $LaCl_3$ at the appropriate temperature was added to the pre-incubated tubes. This was zero time. The dialysis bags containing the liposomes were incubated at the appropriate temperatures for various times. At these times the bags were removed from one tube and placed immediately in another tube containing the same external buffer solution. Aliquots of the dialysate were taken for radioactivity determinations as was a sample from the bags at the termination of the experiments. From the radioactivity determinations the permeability of the liposomes to sucrose could be calculated. Appropriate controls were made for sucrose leakage in the absence of liposomes.

Binding of $^{140}La^{3+}$

^{140}La was obtained as the chloride solution from New England Nuclear (Boston, MA) through Nuclear Facilities (SUNYAB) and was diluted with $LaCl_3$ to form the required stock solution. Counting was performed in Auto-Gamma Scintillation Counter at a Gain setting of 1 MeV full scale, a window setting of 126 and lower level of 337. A decay factor of $\exp(-0.693t/40.2)$ was used to correct for loss in counting due to the decay of ^{140}La, where t(h) indicates the intervals of time between counting of samples.

Binding studies were carried out by equilibrium dialysis of sonicated PS vesicles dialyzed against the standard buffer (without EDTA) containing various concentrations of $LaCl_3$ and NaCl, as described before [2,34], or by direct mixing when precipitates formed rapidly [34,35].

Leakage of carboxyfluorescein

Carboxyfluorescein was obtained from Eastman Kodak and was recrystallized from ethanol/water [28].

The carboxyfluorescein was initially encapsulated in the vesicles at high concentrations, i.e., under conditions of self-quenching [27,28]. Its release was followed by the increase in fluorescence that occurs upon dilution in the external medium.

The carboxyfluorescein-containing vesicles were prepared by hydration and sonication in a solution of 100 mM CF, 0.1 mM EDTA, and 1/10 (v/v) of the standard buffer, adjusted to pH 7.4, separated by passage through a Sephadex G-75 (1.0 × 20 cm) column equilibrated with the standard (0.1 M NaCl) buffer and stored on ice.

Fluorescence of the carboxyfluorescein-containing vesicles was measured with an Aminco-Bowman

spectrofluorometer (excitation 490, emission 550) using a Corning cutoff filter (No. 3-68, ≃520 nm). Complete release was obtained by the addition of Triton X-100 (0.05% v/v). PS concentrations were assayed by phosphate determination [29].

Endothermic phase transitions. The transitions were detected with the Perkin-Elmer DSC-2 differential scanning calorimeter as previously described [2,30].

^{31}P nuclear magnetic resonance

Dispersions of sonicated unilamellar PS vesicles were prepared as described elsewhere [1] in a 90% H_2O/10% ^2H_2O buffer containing 0.1 M NaCl, 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, adjusted to pH 7.4. Equilibrium dialysis against a 90% H_2O/10% ^2H_2O Tris buffer, not containing EDTA, was used to introduce La^{3+} at a specified bulk concentration into the sample solution. Since the volume of dialysate was several hundred times greater than that (1.5 ml) of the sample being dialysed, and since the dialysate solution was changed several times during the course of a 4-h dialysis, the concentration of free La^{3+} in the sample solution could be taken as that in the dialysate. Samples were run in a nitrogen atmosphere. ^{31}P Fourier transform-NMR spectra were taken at 40.5 MHz in the quadrature phase detection mode on a Varian X1-100, NTC TT-100 FT-MNR spectrometer at 32 ± 1°C. Spin-lattice relaxation time measurements employed a standard 180-τ-90 inversion recovery pulse sequence [33] with at least 12 τ values and a delay between acquisitions of 10 s. Proton decoupling with square-wave modulation was used.

Freeze-fracture electron microscopy. Sonicated vesicles of phosphatidylserine were incubated at selected temperatures for thirty minutes with various concentrations of La^{3+}. 0.1 μl of each sample was sandwiched between a pair of copper foils and immediately plunged into liquid propane employing a rapid freezing device similar to that of Ref. 31. The freezing rate was measured to be in excess of 5 000 K/s; therefore, ice crystal growth was prevented even in the absence of cryoprotectants. Freeze-fracture of these samples was performed in a Polaron E 7 500 freeze-etch module at −120°C under a vacuum of $5 \cdot 10^{-7}$ torr or better. Replicas were cast by resistance evaporation, floated off in distilled water, transferred to 1.5% SDS in 30% Clorox, washed three times in distilled H_2O and mounted on bare 460 mesh Hex grids. Representative micrographs were taken with a Siemens 101 electron microscope.

X-ray diffraction

Small and wide angle X-ray diffraction experiments were carried out at selected temperatures. The lipid suspensions were concentrated after incubation by centrifugation at $5\,000 \times g$ for 10 min. The concentrated suspension was placed in a Teflon-lined aluminum cell with a Mica windows. The temperature of the cell was maintained (at the given value) to an accuracy of 0.5°C. Both wide and small angle diffraction lines were recorded on the same film using a Frank camera with slit focusing. The X-ray source was a Jarrell-Ash microfocusing unit with copper target and nickel filters. Each exposure requires about 10 h.

Binding of La^{3+} and other cations to PS · Theoretical analysis

The binding of Ca^{2+}, Mg^{2+}, and Na^+ to PS vesicles has been analyzed [18] by distinguishing between cations tightly bound and those concentrated in the double layer region. Such a distinction is needed because the radioisotope (e.g., ^{45}Ca or ^{140}La) or spectroscopic measurements can only give the total amount of cation sequestered, i.e., both bound and trapped near the negatively charged surface. Refs. 36 and 37 extended the theoretical treatment to the case of solutions containing any combination of ions of the valencies one to three.

The concentration of cations of valency z near the vesicle surface $C_z(0)$, is significantly higher than in the bulk, C_z, due to the negative surface charge on the vesicle. These quantities are related by Boltzmann's equation

$$C_z(0) = C_z \exp(-ze\psi_0/kT) \equiv C_z Y_0^z \qquad (1)$$

in which ψ_0 is the surface potential, e is the magnitude of an electronic charge, k is Boltzmann's factor and T is the absolute temperature. At room temperature the term e/kT equals $(25.2\ \text{mV})^{-1}$. Because of accumulation of cations near the surface, their binding to the head groups becomes much more

pronounced than expected from the binding magnitudes of the binding constants.

All possible types of complexes between La^{3+} and the molecules of the vesicle may exist in the system, however the 1–2 complex, one La^{3+} bound to two phosphatidylserine molecules, seems to most simply account for the data. The binding reactions of Na^+, Ca^{2+}, and La^{3+} can take the form

$$Na^+ + P^- \rightleftharpoons \hat{P}Na$$

$$Ca^{2+} + \hat{P}^{2-} \rightleftharpoons \hat{P}Ca$$

$$La^{3+} + \hat{P}^{2-} \rightleftharpoons \hat{P}La^+$$

where P^- denotes the phosphatidylserine headgroup, $\hat{P}^{2-}$ denotes an adjacent doublet of headgroups, $\hat{P}Na$ and $\hat{P}Ca$ denote the neutral complexes of Na^+ and Ca^{2+} with the phosphatidylserine headgroups and $\hat{P}La^+$ denotes the +1 charged complex. The binding constants for these reactions may be defined by [18,37,38],

$$K_{Na} = [\hat{P}Na]/[Na^+]_s[P^-]$$

$$K_{Ca} = [\hat{P}Ca]/[Ca^{2+}]_s[\hat{P}^{2-}]$$

$$K_{La} = [\hat{P}La^+]/[La^{3+}]_s[\hat{P}^{2-}] \quad (2)$$

where the unit of a complex, e.g., $[\hat{P}La^+]$ is (mol/cm^2), $[La^{3+}]_s$ is the concentration of La^{3+} at the vesicle surface, $[P^-]$ denotes the surface density (mol/cm^2) of phosphatidylserine head groups, while $[\hat{P}^{2-}]$ denotes the surface concentration of adjacent doublets. We define $[\hat{P}^{2-}]$ by

$$[\hat{P}^{2-}] = [P^-]/2 \quad (3)$$

The surface area per phosphatidylserine headgroup is approx. 70 Å^2 [1], hence in the absence of binding $\sigma_0 = (1/70)$ ionic charges/Å^2 $= -6.86 \cdot 10^4$ esu/cm^2. After binding reactions have reached equilibrium the new surface charge density, denoted σ, is

$$\sigma = -e([P^-] - [\hat{P}La^+]) \quad (4)$$

While, in general,

$$\sigma_0 = -e([P^-] + [\hat{P}Na] + 2\,[\hat{P}Ca] + 2\,[\hat{P}La]) \quad (5)$$

From Eqns. 1 to 5

$$\frac{\sigma}{\sigma_0} = \frac{1 - \frac{1}{2}K_{La}Y_0^3[La^{3+}]}{1 + K_{Na}Y_0[Na^+] + K_{Ca}Y_0^2[Ca^{2+}] + K_{La}Y_0^3[La^{3+}]} \quad (6)$$

This provides one equation with to unknowns: σ and Y_0. The solution of the Poisson-Boltzmann equation [18,36–39] provides another equation connecting σ and Y_0 and as has been proven [36] there is a unique solution. The value of K_{La} is chosen from the best fit to the binding data.

Results

Phase transition changes in the presence of La^{3+}, Ca^{2+}, Mg^{2+} and Na^+

In a previous report [8] it was shown that the endothermic peak of phosphatidylserine, which occurs at 9°C in a buffer which contains 100 mM NaCl, is at about 38°C in the presence of La^{3+} or

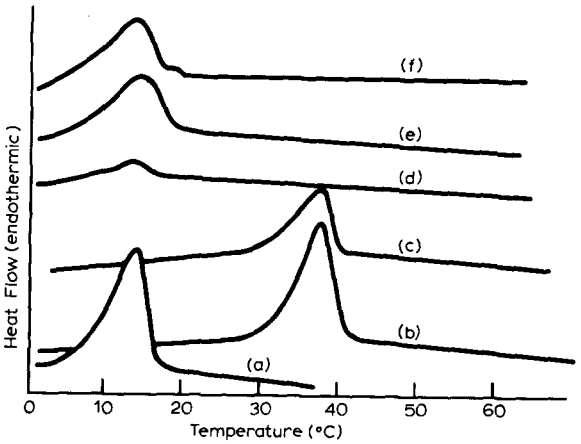


Fig. 1. Differential scanning calorimetry (DSC) of multilamellar PS vesicles in the presence of La^{3+}, Ca^{2+}, Mg^{2+} and 1 000 mM Na^+. The cations were added after the formation of vesicles to the final cation concentrations shown and 1 mM PS at pH 7.4. The samples were given three brief (15 s) bursts of sonication and incubated for 30 min at 37°C. After incubation, the samples were centrifuged at 84 000 × g for 30 min at 20°C. The analysis began 6–12 h after incubation [35]. All samples contained 1 000 mM Na^+ (a), while the other samples contained in addition 0.2 mM La^{3+} (b); 0.5 mM La^{3+} (c); 0.2 mM Ca^{2+} (d); 3 mM Mg^{2+} (e); 0.1 mM Ca^{2+} + 3 mM Mg^{2+} (f).

Gd^{3+}. The results in Fig. 1 illustrate the shapes of the endothermic peaks of multilamellar phosphatidylserine ve
phatidylserine vesicles in the presence of 1 000 mM NaCl and several concentrations of La^{3+}, Ca^{2+} and Mg^{2+}. In the presence of 1 000 mM NaCl the endothermic peak shifts from 9°C to 14°C. Curves (b) and (c) indicate that La^{3+} is effective in shifting the peak to 38°C as in 100 mM Na^+. With 0.1 mM Ca and 3 mM Mg^{2+} (curve f) the peak remains at 14°C whereas in 100 mM NaCl this peak is abolished. With 0.2 mM Ca^{2+} (curve d) the peak is still at 14°C but its height and area are reduced. From the results of Fig. 1 we can conclude that in the presence of 1 000 mM Na^+, the Na^+ could displace a large portion of Ca^{2+} and Mg^{2+} but less of the La^{3+}.

Leakage

The leakage results were obtained by a combination of two complimentary procedures which measure (i) changes in fluorescence intensity following leakage of initially quenched carboxyfluorescein, or (ii) leakage of [^{14}C]sucrose. Before the addition of La^{3+} to the medium the vesicles are stable and their permeability is low. Similarly to the effect of Ca^{2+} or Mg^{2+}, the addition of La^{3+} to the solution results in its binding to the negatively charged groups, which results in promotion of vesicle aggregation [1,40], leakage and fusion. Because of the strong binding capacity of La^{3+} to PS vesicles, it may be expected that small concentrations of La^{3+} would be sufficient to induce vesicle leakage. Curve (b) of Fig. 2 demonstrates that some carboxyfluorescein leakage starts with 0.01 mM La^{3+}, whereas 0.5 mM Ca^{2+} was required [1,21] for a comparable increase in permeability.

Inspection of Fig. 3 indicates a paradoxical result; when the La^{3+} concentration exceeds a certain value, which increases with lipid concentration, the fluorescence intensity drops. We ascribe this effect to a decrease in pH of the lipid suspension when large concentrations of La^{3+} are present. Previous reports [41,42] have already indicated that the fluorescence intensity of carboxyfluorescein is reduced with a reduction in pH. Such a reduction has indeed been measured by us but only in cases where the concentration of La^{3+} in solution approached or exceeded the value required for binding saturation of the lipid. Thus, with 0.038 mM lipid this effect is seen at 0.025 mM La^{3+} [35]. The results of Fig. 4 show the decrease in pH under the following experimental conditions. A suspension of lipid in buffer was added to a carboxyfluorescein solution. No pH changes were recorded and no decrease in fluorescence intensity has been observed (Fig. 4). When La^{3+} was added pH reductions from 7.4 to 6.6 were measured and a decrease in fluorescence intensity was observed (Fig. 4). Raising the pH by the addition of NaOH restored the fluorescence

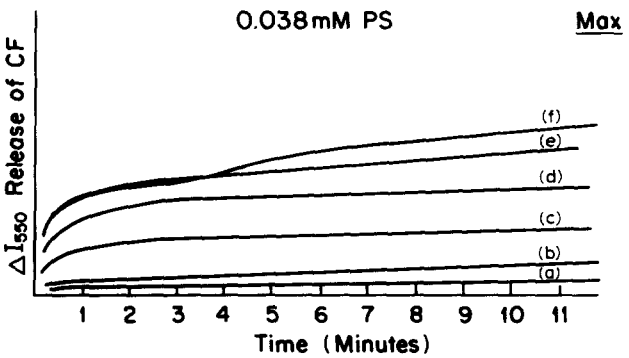


Fig. 2. The extent of carboxyfluorescein release from sonicated PS vesicles (0.038 mM) after addition of La^{3+} with continuous agitation at 24°C, (a) with 0.005 mM La^{3+}, (b) with 0.01 mM La^{3+}, (c) with 0.015 mM La^{3+}, (d) with 0.02 mM La^{3+}, (e) with 0.0225 mM La^{3+}, and (f) with 0.023 mM La^{3+}. Max indicates maximum fluorescence intensity, which is obtained by the addition of 0.2% Triton X-100. In all leakage experiments, the control curves (i.e. when no cations are added) lie on the *x*-axis of the figures [35].

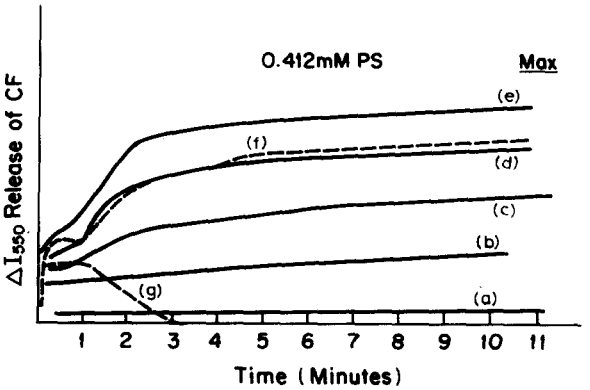


Fig. 3. The intensity of carboxyfluorescein fluorescence released from PS vesicles (0.412 mM) after addition of La^{3+} in a solution of 100 mM NaCl adjusted to pH 7.4 to reach concentrations of (a) 0.05 mM La^{3+}, (b) 0.1 mM La^{3+}, (c) 0.125 mM La^{3+}, (d) 0.145 mM La^{3+}, (e) 0.15 mM La^{3+}, (f) 0.175 mM La^{3+}, and (g) 0.3 mM La^{3+}.

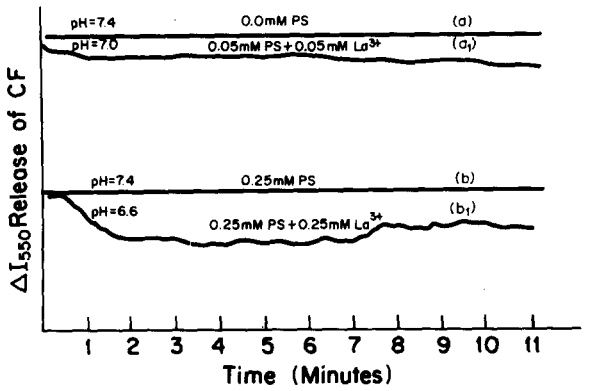


Fig. 4. Decrease in pH of solutions upon the addition of La^{3+} to PS vesicles as monitored by carboxyfluorescein fluorescence decrease and by direct pH measurements. A suspension of vesicles in buffer was added to a carboxyfluorescein solution (0.05 mM) followed by the addition of La^{3+}. (a) 0.05 mM PS alone, (a_1) 0.05 mM PS + 0.05 mM La^{3+}, (b) 0.25 mM PS alone, (b_1) 0.25 mM PS + 0.25 mM La^{3+}.

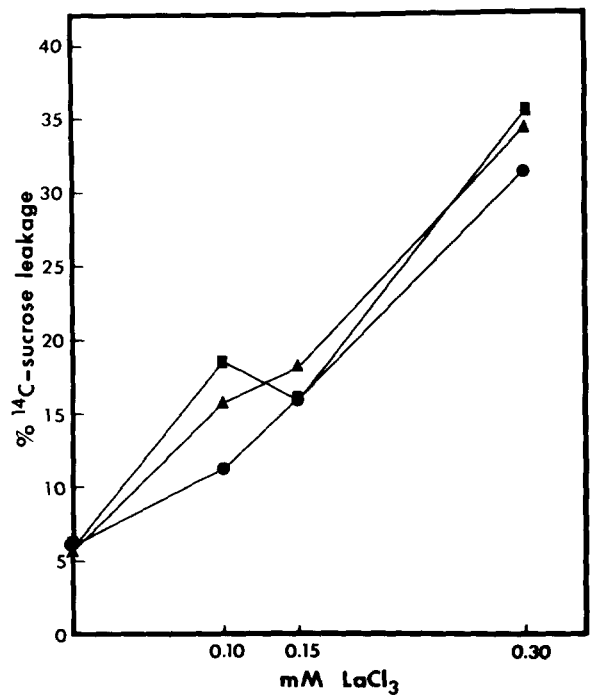


Fig. 5. Release of [^{14}C]sucrose due to La^{3+}. Abscissa: Concentration of La^{3+} in dialysis bags at zero time. Ordinate: [^{14}C]sucrose leaked from dialysis bags, normalized as a percentage of non-entrapped [^{14}C]sucrose leaked from control dialysis bags, during 10 min incubation at the following temperatures 24°C (●——●), 38°C (■——■), 50°C (▲——▲). The data shown are the means from two experiments.

intensity to its initial value. It may be noted that in the absence of the lipid, the addition of La^{3+} did not cause any changes in the fluorescence intensity of carboxyfluorescein.

The [^{14}C]sucrose leakage result in Fig. 5 provide another direct demonstration of the fact that an increase in La^{3+} concentration gives an increase in leakage from sonicated phosphatidylserine vesicles.

Effect of temperature. The results in Fig. 6 demonstrate that a larger rate of leakage of carboxyfluorescein occurs at 38°C, i.e., at the phase transition temperature of the PS/La complex, than at 24°C or at 50°C. This result confirms the proposal [1,3, 43] that maximum destabilization of the vesicles is expected at the phase transition temperature. In Fig. 5 we present the sucrose leakage percent divided by the percent of leakage in control experiments which give the leakage through the bag of sucrose in solution. It should be noted that the sucrose release measured is the outcome of two processes, release and then diffusion through the bag. As the diffusion coefficient increases sharply with temperature, the temperature dependence of the leakage could be masked. No marked increase in the sucrose permeability at 38°C was observed, although the method used for the sucrose permeability

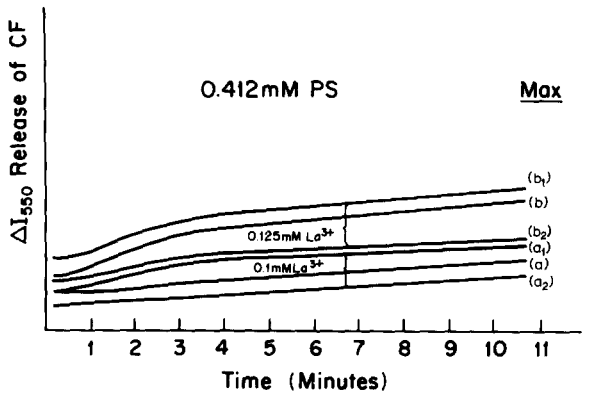


Fig. 6. The effect of temperatures below and above the T_c of La/PS complex on the extent of carboxyfluorescein release from sonicated PS vesicles (0.412 mM) upon the addition of La^{3+} to reach the following initial concentrations in 100 mM NaCl buffer adjusted to pH 7.4. The temperatures were 24, 38 and 50°C. (a) 0.1 mM La^{3+} at 24°C, (a_1) 0.1 mM La^{3+} at 38°C, (a_2) 0.1 mM La^{3+} at 50°C, (b) 0.125 mM La^{3+} at 24°C, (b_1) 0.125 mM La^{3+} at 38°C, and (b_2) 0.125 mM La^{3+} at 50°C.

measurement is capable of detecting changes in the permeability at or near the phase transition temperatures [25]. Thus the data of both carboxyfluorescein and sucrose permeability measurements suggest that the peak in permeability at 38°C is relatively small.

La binding to PS

The results are divided into three groups. In Table IA we present results for very low La^{3+} solution concentration, from 0.0043 mM to 0.0275 mM, in the presence of 100 mM NaCl. In these solutions the degree of leakage, aggregation and fusion of sonicated PS vesicles is minimal. These cases correspond to a relatively simple behavior and are explained within the framework of a modified Gouy Chapman equation, Eqn. 6. We have assumed that La binds only on the external surface of the vesicles in this range of concentrations.

The second range is illustrated in Table IB and corresponds to situations where a significant degree of leakage occurs and therefore it has been assumed that La binds to both monolayers of the vesicle. In this range there is some degree of vesicle aggregation and probably fusion as well. The results in Table IC illustrate the third range for which the conditions have been chosen to maximize structural changes of PS vesicles by using high concentrations of La^{3+} (>0.5 mM) and a temperature of incubation of 38°C, which corresponds to the phase transition of the La/PS complex. The striking feature of these results is that with an increase in La^{3+} concentration of the binding ration La/PS progressively increases beyond the stoichiometric ratio of 1/3 to a ratio of 1/2. The conditions described in Table IC bring about a large degree of aggregation, leakage and structural changes, of PS vesicles as is described in other parts of this work (see Fig. 7).

Because of the limiting 1/2 binding ratio of La/PS, we have attempted to model the binding by a 1-2 complex, one La^{3+} bound to two PS molecules, using a binding constant of 3 M^{-1} for La^{3+}. With the limited data it is not possible to unambiguously conclude that the predominant La^{3+}-PS complex is 1-2. The data in Table IA could be fit nearly as well using 1-1 binding and a binding constant of 1 M^{-1} or 1-3 binding and a binding constant of

TABLE IA

EXPERIMENTAL AND CALCULATED BINDING RATIOS OF La/PS FOR SONICATED PS VESICLES

The bulk La^{3+} concentrations were determined directly using $^{140}La^{3+}$ after 14 h of dialysis as described in Materials and Methods [2,34].

		La^{3+} bulk concentration (mM) with 100 mM Na^+			
		0.0043	0.0087	0.0185	0.0275
Experimental ratio La/Ps *		0.017	0.030	0.062	0.12
Calculated total La/PS	(1-2 binding) **	0.021	0.035	0.056	0.07
	(1-3 binding) ***	0.023	0.040	0.065	0.08
Calculated tightly bound La/PS	(1-2 binding)	0.019	0.033	0.052	0.065
	(1-3 binding)	0.022	0.038	0.060	0.074
Calculated total Na/PS	(1-2 binding)	0.87	0.83	0.77	0.73
	(1-3 binding)	0.87	0.82	0.74	0.70
Calculated tightly bound Na/PS	(1-2 La binding)	0.59	0.56	0.52	0.49
	(1-3 La binding)	0.58	0.54	0.48	0.45
Surface potential, ψ_0 (mV)	(1-2 binding)	−76	−74	−72	−70
	(1-3 binding)	−76	−75	−73	−72

* It is assumed that only the outside monolayer of the vesicle was exposed to La^{3+} binding, i.e., 0.66 of the total PS present [2].

** The calculated values assume an area of 70 Å per PS headgroup, and a temperature of 20°C. The intrinsic binding constant used for 1-2 binding of La/PS is K_{La} = 3 M^{-1}. For sodium binding to PS K_{Na} = 0.8 M^{-1} [18].

*** For 1-3 binding K_{La} = 5 M^{-1}.

TABLE IB

La^{3+} BINDING AT INTERMEDIATE CONCENTRATIONS

The bulk La^{3+} concentrations were determined directly using $^{140}La^{3+}$. In the 300 mM Na^+ cases La^{3+} was determined after 14 h of dialysis as described in Materials and Methods [2,34].

Bulk La^{3+} (mM)	Bulk Na^+ (mM)		Ratio La/PS sequestered	
	Int.	Ext.	Exp. **	Calc. ***
0.016	100	300	0.086	0.064
0.026	100	300	0.11	0.082
0.027	300	300	0.058	0.083
0.044	300	300	0.09	0.103
0.26	100	500	0.11	0.13
0.43	100	500	0.15	0.15
0.61	100	500	0.21	0.16

* The vesicles were sonicated in either 100 mM or 300 mM Na^+, as noted (Int., interior (initially)), and then incubated in 300 mM or 500 mM Na^+ as noted (Ext., exterior).

** It is assumed that the La^{3+} was in contact with all of the lipid.

*** The 1-2 binding constant is 100 M^{-1}.

5 M^{-1}. Table IA also contains the calculated values for Na^+ bound to the PS, the amount of cations trapped in the double layer of the vesicles and the surface potential. It is evident that most of the La^{3+} associated with the vesicles is tightly bound, whereas only approx. 60% of the total Na^+ sequestered is tightly bound.

For the systems in Table IB there is a significant aggregation and fusion, hence the binding equations cannot be expected to hold with the same binding constant which was obtained from disperse systems. For example, merely bringing two vesicles together will increase the amount of La/PS sequestered due to the increased surface potential. As shown in Table IB an approximate fit of the experimental binding data is obtained by using a value of 100 M^{-1} for the 1-2 binding complex.

TABLE IC

RESULTS OF La BINDING TO PS AT HIGH CONCENTRATIONS OF La^{3+} AND AT 38°C

The La^{3+} concentrations are the values initially added. All experiments are in the presence of 100 mM NaCl.

Bulk La^{3+} (mM)	La/PS sequestered
0.7	0.31
1.0	0.41
2.0	0.43
17.0 *	0.51

* This case was incubated for 2 hours at 38°C and then for 12 hours at room temperature. The other cases were incubated for 2 hours.

^{31}P chemical shifts and spin-lattice relaxation

As the concentration of free La^{3+} is increased, the ^{31}P-NMR linewidths of the PS small unilamellar vesicles sample increased. Only one resolved ^{31}P line was observed. This situation contrasts with results from similar experiments with Ca^{2+} or Mg^{2+} [44] where two ^{31}P lines were resolved: one corresponding to phosphate groups on the inside of the vesicle where there was no divalent cation and other line corresponding to the phosphate on the outside of vesicle, in the presence of the divalent cations. At the low concentration of La^{3+} used, 0.01 to 0.04 mM, the binding ratio of La/PS varies from 0.04 to 0.12–0.15 (Tables IA and IB). In the lower concentration range the ^{31}P shift probably cannot be detected because of the low binding ratio. For the case of 0.04 mM La^{3+} the binding ratio corresponds to the lowest binding ratio of Ca/PS for which a difference in chemical shifts between inner and outer

TABLE II

^{31}P-NMR RELAXATION RATES FOR PS VESICLES IN THE PRESENCE OF La^{3+}, Ca^{2+} OR Mg^{2+}

For experimental conditions, see text.

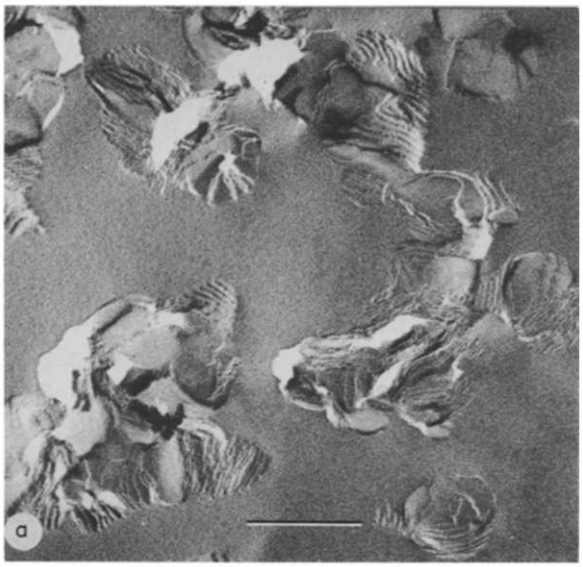
Concentration (mM)				$1/T_1$ (s^{-1})
PS	La^{3+}	Ca^{2+}	Mg^{2+}	
15	0	0	0	0.529 *
15	0	0.40	0	3.3 *
30	0	0	2.0	0.93 *
15	0.01	0	0	0.73 ± 0.01 **
15	0.02	0	0	1.83 ± 0.08 **
15	0.04	0	0	3.63 ± 0.18 **

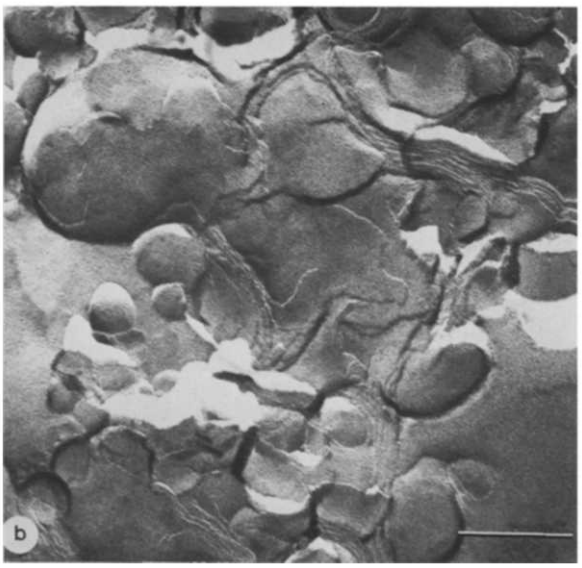
* Ref. 44.

** Specified error limits correspond to those given (±1 S.D.) for a non-linear, iterative, least-squared fit of the observed integrated intensities to the magnetization decay curve, $M_z(t) = M_0(1 - 2\exp(-\tau/T_1))$.

phosphate groups of PS was measured [2,18,44]. However, at this La^{3+} concentration the leakage is significant and therefore La^{3+} can permeate into the interior of vesicles. Thus both inside and outside phosphate groups would be exposed to La^{3+} and ^{31}P lines for both kinds of phosphate would be shifted by about the same amount.

The effect of La^{3+} on the ^{31}P spin-lattice relaxation rates ($1/T_1$) is pronounced, as can be seen from the values given in Table II. For comparison, the previously reported ^{31}P spin-lattice relaxation rates [44] produced by Ca^{2+} and Mg^{2+} are also given in Table II. It is clear that an approx. 10-fold higher concentration of Ca^{2+} than La^{3+} is required to produce the same relaxation rate increase. If the fraction of PS headgroups complexed to Ca^{2+} is about the same as that for La^{3+} at the same bulk concentration of each ion, then it is clear that the intrinsic relaxation rate of the headgroup phosphorus is much greater in the La^{3+}-PS complex than in the Ca^{2+}-PS. Accordingly, one can argue, that the La^{3+}-PS headgroup complex is more rigid and/or more PS headgroups are effected by the complexation than is the case for the Ca^{2+}-PS headgroup complex.





Morphological data

Prior to the addition of La^{3+}, PS at room temperature and above is in a liquid crystalline phase. Multilamellar vesicles of PS show a diffuse low angle band with a spacing of approx. 70 Å, indicating the lamellar repeat is irregular.

Incubation of small unilamellar vesicles in the presence of 1.0 mM La^{3+} for 30 min at 21°C yields multilamellar vesicles that are aggregated; many of which form flattened stacks. The diameters of these

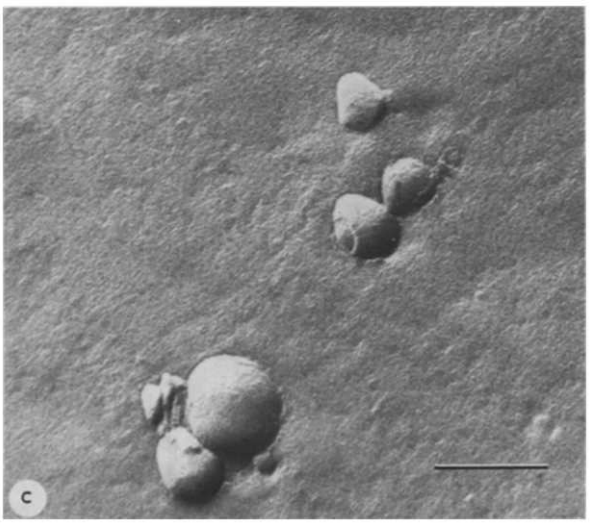


Fig. 7. Freese-fracture micrograph of PS vesicles. Bar = 200 nm. (a) Incubated in 1.0 mM La^{3+} at 21°C for 30 min. (b) Incubated in 1.0 mM La^{3+} at 39°C for 30 min. Freeze-fracture micrograph of PS vesicles incubated. (c) Incubated in 0.2 mM La^{3+} at 39°C for 30 min.

vesicles range between 300 and 3 000 Å (Fig. 7a). When incubated at 39°C, the resultant structures are large, aggregated and sometimes flattened multilamellar structures (Fig. 7b). Incubation at 50°C yields similar but larger multilamellar structures than those seen at an incubation temperature of 39°C (data not shown). The X-ray diffraction data for these samples are given in Table III. The addition of 1.0 mM La^{3+} has clearly fused the small unilamellar vesicles into multilamellar vesicles, and at the same time converted the PS from the fluid state to a gel state. The molecular reorganization process seems to proceed more thoroughly at 50 and 39°C than at 24°C, and results in a larger multilamellar vesicles with closer interlamellar packing.

Incubation of PS small unilamellar vesicles at 21, 39, and 50°C in the presence of 0.18 mM La^{3+} yields similar structures at all incubation temperatures. The structures appear as small aggregated vesicles (Fig. 7c). At this La^{3+} concentration, fusion between small unilamellar vesicles seems to have been initiated but the formation of multilamellar vesicles is not yet predominant. X-ray diffraction data show the multilayer structures formed have a shorter (59 Å) lamellar repeat than the multilamellar vesicles formed in the absence of La^{3+} (70 Å), and the acyl chain packing is that of a hexagonal gel state.

TABLE III

X-RAY DIFFRACTION LAMELLAR REPEAT SPACING: La^{3+} AND TEMPERATURES

The vesicles were initially suspended in the 100 mM Na^+ buffer, see Materials and Methods.

La^{3+} (mM)	Incubation temp. (°C)	Lamellar repeat (Å)
1.0	21	61 ± 1
1.0	39	56 ± 1
1.0	50	56 ± 1
0.18	21	59 ± 1
0.18	39	59 ± 1
0.18	50	59 ± 1

Discussion

Correlation between cation binding and structural changes

It has been pointed out [9–11,45] that La^{3+} and Ca^{2+} share some specificity in their binding to several biological surfaces. In our study we have found that La^{3+} has a much stronger interaction with phosphatidylserine vesicles than does Ca^{2+}. The structural changes to the PS vesicles (as shown by leakage, DSC, freeze-fracture and X-ray diffraction) are induced by La^{3+} at about one-tenth the concentration required of Ca^{2+}. This ratio agrees also with direct binding studies using both Ca^{2+} and La^{3+} [8,35].

We will first examine the binding behavior at low La^{3+} concentrations (Table IA), i.e., when neither leakage nor fusion occur, and compare it with the case of low Ca^{2+} concentrations. In the presence of 0.0275 mM La^{3+} the binding ratio La/PS is 0.12 (Table IA), i.e., slightly less than 0.14, which is the Ca/PS ratio [2] in the presence of 0.104 mM Ca^{2+}. A naive comparison of these experimental results would suggest that the binding strength of La^{3+} to PS is 3-times larger than that of Ca^{2+}. However, it is essential to recall that the surface concentrations of these cations are larger than their bulk concentrations by factors of $\exp(-3e\psi_0/kT)$ for La^{3+} and $\exp(-2e\psi_0/kT)$ for Ca^{2+}. In these two cases, 0.0275 mM La^{3+} and 0.104 mM Ca^{2+} in 100 mM Na^+, the calculated surface potentials (Table IA and Ref. 18) are nearly equal, at −70 mV, which implies that the surface concentration of La^{3+} is about 4-times larger than the Ca^{2+} surface concentrations. In fact, the intrinsic binding constant of La^{3+} to PS turns out to be 5 M^{-1} for 1-3 binding or 3 M^{-1} for 1-2 bindings, whereas in the case of Ca^{2+} it is 35 M^{-1} in the low concentration range.

The fitting of calculated values of La/PS ratios to the experimental values in Table IB requires a 1-3 binding constant of 200 M^{-1} [35] or a 1-2 binding constant of 100 M^{-1}. The La^{3+} concentrations in Table IB are above the threshold for leakage and fusion of the small PS vesicles. This higher binding constant, together with the onset of bilayer destabilization, indicate the emergence of a new, distinct La-PS complex at these higher La^{3+} concentrations. It is of interest to note that NMR studies have shown that La^{3+} and other lanthanides induce con-

formational changes in phosphatidylcholine [46] and lysophosphatidylcholine [47]. The results of Table IC, wherein a final ratio of 0.5 La/PS is reached at high La^{3+} concentrations (>2 mM), may be construed to indicate the presence of yet a third type of complex, although there are insufficient data on this point. It is of interest, though, that a study of the interaction of Mn^{2+} with PS [48] has reported the release of an amino proton from PS. The above study noted that such release was not detected with Ca^{2+} in the presence of 100 mM NaCl, although a significant H^+ release occurred when Ca^{2+} was added to PS dispersions at pH $\simeq$ 6.1 in the absence of NaCl. We did not detect pH changes even in the presence of 50 mM Ca^{2+}, whereas the results in Fig. 4 indicate some pH changes in the presence of 0.05 mM La^{3+}. Although we do not have data on the surface charge or on the amount of H^+ released in the presence of large La^{3+} concentrations, it may be that La^{3+}, like Mn^{2+}, causes the release of an amino proton from PS.

The previous DSC results with Ca^{2+}, in dialysis experiments where Ca^{2+} ionophores were employed [34], indicate that in the presence of 0.25 mM Ca^{2+} (binding ratio Ca/PS approx. 0.25) the PS peak is shifted to very high temperatures. In contrast, the PS peak is essentially unshifted in the presence of 0.1 mM Ca^{2+} (binding ratio Ca/PS is 0.14), even when the buffer contained the Ca^{2+} initially. That is when only 28% of the PS is bound to Ca^{2+} the PS peak remains, but when 50% of the PS is bound by Ca^{2+} the peak disappears, or is shifted to very high temperatures, even though 50% of the PS is not directly complexed to the Ca^{2+}. This type of cooperativity is also indicated by La^{3+}. With 0.1 mM La^{3+} in 100 mM Na^+, the calculated binding ratio is 0.23 La/PS and under these conditions the endothermic PS peak shifts to 38°C, i.e. the phase transition temperature (T_c) of the La-PS complex. The results of Refs. 8 and 35 indicate the existence of the La/PS endothermic peak in the absence of 0.2 mM La^{3+} + 0.2 mM Ca^{2+} + 100 mM Na^+. These results are explained by the calculated values in Table IV which show a binding ratio of 0.23 for La/PS and 0.04 for Ca/PS. The binding ratio of Ca/PS in the presence of 0.2 mM Ca^{2+} + 1 000 mM Na^+ is only 0.02, while in Fig. 1 we see that the endothermic peak is the same as that of PS in the presence of 1 000 mM Na^+.

TABLE IV

CALCULATED CATION BINDING RATIOS FOR DSC RESULTS

These cases correspond to DSC results given in Fig. 1 of this work (for 1000 mM Na^+) and in Fig. 1 in Refs. 8 and 35 (for 100 mM Na^+). M^{2+} denotes the appropriate divalent cation. M/PS denotes the calculated binding ratio for the indicated divalent cation. The binding constants used are 0.8 M^{-1} for Na^+, 75 M^{-1} for Ca^{2+}, 20 M^{-1} for Mg^{2+} [4,21] and 100 M^{-1} for the 1-2 binding of La^{3+}. The results are close to those of Ref. 35 where a 1-3 binding of La^{3+} was employed with a binding constant of 200 M^{-1}.

Cation concn. (mM)			Cation binding ratios[c]		
Na^+	La^{3+}	M^{2+}	Na/PS	La/PS	M/PS
100	–	–	0.63	–	–
100	0.1	–	0.15	0.23	–
100	0.2	–	0.12	0.25	–
100	0.5	–	0.09	0.27	–
100	0.2	+0.2 mM Ca^{2+}	0.11	0.23	0.04
100	0.2	+3.0 mM Mg^{2+}	0.09	0.17	0.13
100	–	+0.2 mM Ca^{2+}	0.24	–	0.25
1000	–	–	0.70	–	–
1000	0.2	–	0.61	0.05	–
1000	0.5	–	0.53	0.08	–
1000	–	+0.2 mM Ca^{2+}	0.67	–	0.02
1000	–	+3.0 mM Mg^{2+}	0.60	–	0.06

Fusion and the other structural changes in sonicated phosphatidylserine vesicles are induced by divalent cations such as Ca^{2+} and Mg^{2+} or by a trivalent cation such as La^{3+}. However, monovalent cations such as Na^+ stabilize these vesicles although they neutralize a significant fraction of their charges [21] and induce their aggregation [21,22]. The explanation is that monovalent cations can bind to the phospholipid headgroups without introducing spatial constraints, possibly as outer sphere type complexes. On the other hand the binding of multivalent cations requires some structural rearrangement of the molecules on the surfaces in such a way as to optimize the surface charge neutralization. Such a reduction in the free energy of the system favors a crystalline structure which is seen by the DSC results (Fig. 1) as well as by X-ray diffraction (Table III) and freeze-fracture (Figs. 7a–c). At a certain degree of binding by the multivalent cations, which corresponds to approximately 0.35 for M^{2+}/PS (Ca^{2+} or Mg^{2+} [21]) and to 0.1 for La^{3+}/PS, the small

vesicles become destabilized as is seen by leakage experiments, and the final outcome is a crystalline structure. At this stage of corresponding charge neutralization it becomes progressively more difficult to enable optimal sterical fitting of pairs or triads of headgroups to divalent or trivalent cations, respectively, and rearrangement of the molecules composing the surface occurs, which results in leakage and fusion.

All the experimental methods employed in this study demonstrate the existence of a sequence $La^{3+} > Ca^{2+} > Mg^{2+} > Na^{+}$, regarding binding capacity as well as the potential to induce fusion and structural changes. The NMR results indicate that for the same degree of cation binding to the headgroup there is a larger enhancement of the ^{31}P spin-lattice relaxation rates with La^{3+}, in accord with the above sequence. This enhancement of relaxation rate, which has been measured under conditions of minimal fusion, already reflects significant changes in the membrane, i.e., enhancement of local rigidity or microviscosity. The trivalency of La^{3+} is probably an important factor in causing a significant local rigidity and in inducing leakage and fusion of the small phosphatidylserine vesicles at a smaller binding ratio than in the case of the divalent cations.

The term fusion has been widely used in this study although our experiments have only provided direct demonstration of bilayer structural changes. A recently developed fusion assay [5,6] which measures the internal mixing of the vesicle contents indicates that the structural changes induced by La^{3+} reflect membrane fusion events (Düzgünes, N., personal communication to S.N.).

Effect of temperature

Our results demonstrate that both leakage of CF and structural changes occur faster at the phase transition temperature, T_c, of phosphatidylserine in the presence of La^{3+}. Incubation for 2 h at 38°C resulted in larger La/PS binding ratios than at room temperature (Tables IB and IC). These results are remarkable in view of the fact that the process of vesicle-vesicle fusion is complex and involves several stages such as a close approach, destabilization and merging of the membranes. The process of leakage involves destabilization and diffusion. The diffusion coefficients of molecules increase with temperature. Hence we can anticipate that the process of merging and rearrangement of molecules in fusing membranes would be faster when the temperatures are increased. If we ignore the possibility of reversible aggregation [22,23] in La^{3+}-induced fusion of vesicles we would anticipate the rate of aggregation to increase with temperature [21]. Thus of all the stages of the La^{3+}-induced fusion of phosphatidylserine vesicles their destabilization is the only process which is optimal at the phase transition temperature of the La-PS complex. Therefore we may conclude that this process plays a very important role in this overall fusion. The explanation is [1,49] that at the phase transition temperature the vesicles are in a state of transient instability from which structural changes can proceed rapidly. Studies with other phospholipids showed that maximum fusion occurred in a narrow temperature range around T_c [43]. Recent studies [3] demonstrated that the increase in the size of phosphatidylserine vesicles in the presence of Ca^{2+} was largest at a particular temperature, which increased with Ca^{2+} concentration, and hence might be the T_c of the PS/Ca at a prefusion stage. From our analysis we would not anticipate, in general, that a maximum rate of fusion should occur at the phase transition temperature. Whenever the maximum rate of fusion does occur at the T_c, it is an indication that the process of vesicle destabilization plays an important role. This destabilization could also promote vesicle aggregation, because the formation of surface defects or breakage is expected to reduce the potential barrier for close approach, as has been proposed for the case of Ca^{2+} induced fusion of phosphatidylserine vesicles which is promoted in low ionic strength [21].

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