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An ionotropic phase transition in phosphatidylcholine: cation and anion cooperativity *

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Evidence is presented for cooperative interaction between cations and anions specifically bound to dimyristoylphosphatidylcholine (DMPC). The cooperativity is with regard to an ion-induced (ionotropic) phase transition for the lipid and is signalled by a change in the luminescence from bound Tb^{3+}. The intrinsic binding of Tb^{3+} to DMPC was determined from equilibrium dialysis experiments, using conventional methods to correct for electrostatic contributions. Preliminary results demonstrate great potential for infrared spectroscopy as a means to relate these Tb^{3+} luminescence studies to experiments involving less tractable cations. This work provides insight into the role of bound ions in modifying lateral phase behavior in phospholipid membranes.

Introduction

Specific binding of ions to phospholipid membranes has been widely studied because of its effect on the membrane surface charge and the lipid phase behavior. The surface charge is integral in defining concentrations of ions near the membrane surface [1] and the transmembrane potential [2]. These factors determine the membrane states responsible for nerve transmission and chemical energy transduction in mitochondria and chloroplasts. Lipid phase behavior influences membrane permeability [3], fluidity and packing of the membrane components; fluidity and packing have been shown to affect the activity of integral proteins [4]. The results presented here indicate that cations and anions may work cooperatively in modifying the phase behavior of lipid membranes, at least those containing phosphatidylcholine.

Phosphatidylcholine is a major lipid class in most biological membranes. Although it is zwitterionic, cations bind with roughly the same intrinsic affinity as to anionic phospholipids [5–9]. Several anions have also been shown to specifically bind to vesicles made of this lipid [10,11]. Since artificial membranes prepared from phosphatidylcholine have no intrinsic surface charge,

* Data supplementary to this article are deposited with, and can be obtained from: Elsevier Science Publishers B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/369/73624/902 (1987) 53. The Supplementary information includes: Fig. 7. Phosphate stretch region of the cardiolipin infrared spectrum. (2) Appendix I.

Abbreviations: DMPC, dimyristoylphosphatidylcholine; FTIR spectroscopy, Fourier transform infrared spectroscopy; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetate; DPA, dipicolinic acid.

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even low levels of bound ions have a great effect on the surface potential and the apparent binding of other ions. There is a very strong anticooperativity in the binding of ions with a similar charge and a strong cooperativity between cations and anions, as expected from purely coulombic arguments [11]. Cation binding has also been shown to alter the phase state of phosphatidylcholine membranes [6,12–15]. In this work, the luminescence from Tb^{3+} was used to monitor changes in the phase state of dimyristoylphosphatidylcholine (DMPC) induced by the binding of this trivalent cation.

The trivalent lanthanides, Tb^{3+} and Eu^{3+}, have been used as luminescent probes of cation binding sites in several biochemical systems [16,17]. Most of the work with lanthanide-lipid complexes, however, has exploited the paramagnetic properties of the cation and their effect on the lipid NMR spectrum [5,20,21], with the exception of two very recent studies [18,19]. The Tb^{3+} luminescence experiments presented here are complementary to the NMR studies in that the luminescence reflects subtle changes in the cation environment which are not apparent in the lipid NMR spectra. Preliminary work indicates that infrared spectroscopy will prove useful in describing the structural changes in the lipid bilayer which are indicated in the Tb^{3+} luminescence experiments.

There has been some disagreement over the particular model to be used for cation binding to phosphatidylcholine [5,6,20]. Equilibrium dialysis experiments were employed in an attempt to resolve this question, at least for the case of Tb^{3+} binding. A simple binding model with conventional electrostatic corrections was found to be adequate.

Materials and Methods

Dimyristoylphosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids and used as received. $TbCl_3$ and all other salts were dried to constant weight in a vacuum oven and concentrations of stock solutions were calculated from the weight of solute. DMPC was suspended in the appropriate electrolyte solution using a 300 watt ultrasonic dismembrator (Fisher) with a 25 ml jacketted bath attachment. The DMPC solution was contained in a glass vial and submerged in the sonicator bath, which was maintained at 32–35 °C with a water circulator. One hour sonication at 75% full power was required to obtain optically clear solutions using 2.0 mM DMPC. The solutions were unbuffered but the pH was consistently within the range of 5.7–6.3. All glassware was cleaned, soaked in an EDTA solution and thoroughly rinsed with distilled water before use.

Luminescence studies were performed on a spectrofluorometer equipped with double quarter meter monochromators, a 450W Xe lamp, and photon counting detection(Spex Industries). Quartz cells were used which have a 4 mm excitation path to reduce the inner filter effect produced by vesicle scattering. Excitation at 307 nm (1.6 nm band pass) was found to optimize emission and minimize the effect of scattered light at 545 nm, the spectral region of interest. All spectra were corrected for the throughput of the emission monochromator and the response of the detector. The cell temperature was maintained by a jacketted turret and circulating bath. A digital thermometer was used to measure the cell temperature with a precision of 0.1 °C.

Tb^{3+} dialysis studies were performed with membranes having a 6000 molecular weight cutoff in 1 ml teflon chambers (Spectrum). The chambers were submerged in a water bath for temperature control and continuously rotated to accelerate equilibration. Because intact DMPC vesicles are impermeable to electrolytes, the DMPC solution was periodically removed from the chamber, transferred to its vial, sonicated for 20 min to equilibrate the internal vesicle volume and then returned to the dialysis chamber. Three such sonication cycles were found to be adequate over a 24 h period, given the accuracy of the experiments. Following equilibration, the DMPC was removed and an Tb^{3+} emission spectrum collected. The total Tb^{3+} concentration in both the DMPC solution and the lipid free solution was measured by a dipicolinic acid (DPA) assay [22]. Tb^{3+}-DPA complexation greatly enhances the Tb^{3+} emission as a result of absorption by the DPA. Aliquots of the dialysis solutions were added to 2 ml of 5 mM DPA with 2% by weight Triton X-100. The latter was used to disrupt the DMPC vesicles. Tb^{3+} concentrations were calculated from the measured

emission of the DPA complex using a calibration curve generated with a standard Tb^{3+} stock solution, treated in an identical manner. The calibration curve was fit with a cubic equation to deal with the curvature arising from the multiplicity of the Tb^{3+}-DPA complexes formed.

Infrared spectra were collected on a Mattson Cygnus 25B spectrometer using a jacketted transmission cell, ZnSe windows and 25 μm Teflon spacers. This short path with ZnSe windows does give rise to spectral artifacts due to window reflection. However, the artifacts were negligible after subtracting the water spectrum from lipid spectra and the low water solubility of ZnSe is highly desirable in these studies. Again, the temperature was controlled with a refrigerated circulating bath and measured at the cell with a digital thermometer. The DMPC samples were prepared in the appropriate aqueous solution as 15% by weight DMPC with several successive heating and cooling cycles. For each spectrum, 500 scans were coadded using a maximum optical retardation of 0.52 cm. The resultant interferograms were triangularly apodized and transformed with a final resolution of 2 cm^{-1}. The spectral point density is 0.25 cm^{-1}.

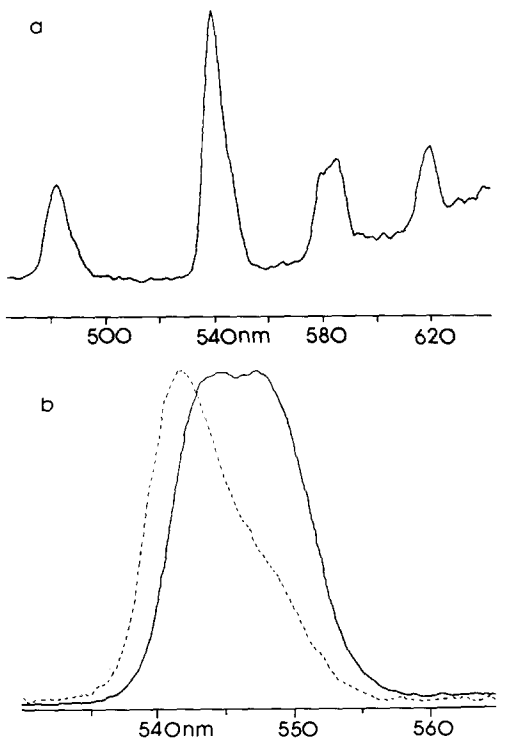

Fig. 1. Tb^{3+} emission spectra: (a) aquo-Tb^{3+} using excitation at 340 nm. (b) The 545 nm emission band using 307 nm excitation: the broken line is aquo-Tb^{3+} and the solid line is 0.1 mM $TbCl_3$ in 2.0 mM DMPC.

Results

Tb^{3+} has several absorption bands in the ultraviolet spectrum and four well resolved emission bands in the visible region: 490, 545, 590 and 620 nm (Fig. 1a). The positions of these bands are relatively invariant with changes in coordination, although the band shape does vary with the symmetry of the ligand field [16,17]. This can be seen for the 545 nm band of aquo-Tb^{3+} and DMPC-Tb^{3+} (Fig. 1b). There are 8–9 water molecules defining the coordination sphere in the aquo form [16]. Up to six of these water molecules are displaced when the lanthanide binds to phospholipids [18], with two phosphate moieties involved in the new complex [20]. Solutions containing DMPC vesicles are highly scattering, which interfers with observation of the full emission spectrum. Consequently, the 545 nm band was chosen for optimization in these experiments primarily for its greater intensity.

Tb^{3+} binding to DMPC not only alters the band shape but results in an increase in the emission efficiency of up to three orders of magnitude relative to the aquo species (vide infra). There are two likely causes for this enhanced emission: displacement of coordinated water molecules which quench Tb^{3+} emission, and sensitization of absorption by the coordinated DMPC moieties [16]. Complete displacement of the water molecules by ligands which do not quench the Tb^{3+} emission may result in a little more than one order of magnitude increase in the steady state emission intensity but not three. It is more likely the changes in Tb^{3+} emission efficiency result from DMPC induced changes in the absorption properties. This issue is currently being investigated. Fortunately, the particular causes of the enhancement need not be understood for the effect to be useful.

Titration of DMPC with Tb^{3+} results in bimodal behavior for the Tb^{3+} emission, as shown in Fig. 2a. The rise and fall in the emission with increasing Tb^{3+} concentration can be due to either an ionotropic phase transition for the Tb^{3+}-DMPC

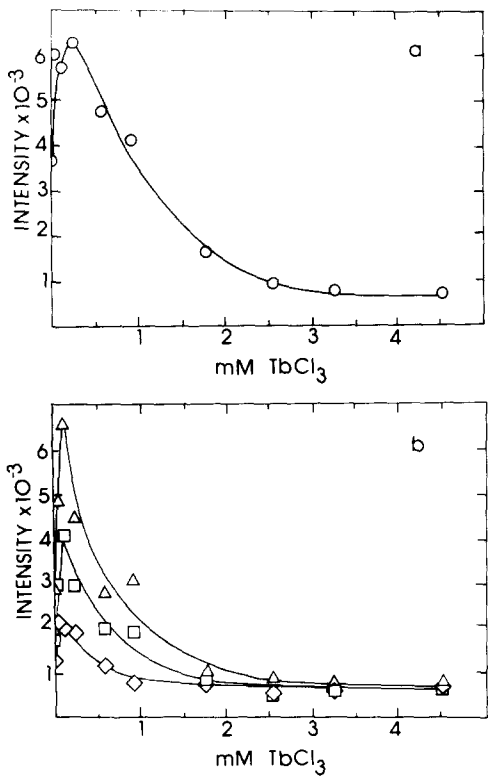

Fig. 2. Tb^{3+}-DMPC titrations: (a) 2 mM DMPC with no additional electrolyte. (b) 2 mM DMPC in 0.010 M (triangles), 0.100 M (squares) and 1.00 M NaCl (diamonds). The intensity is the area of the 545 nm band for the sample divided by the area for a $TbCl_3$ reference spectrum obtained the same day. Each point represents a separate DMPC sample prepared by mixing appropriate volumes of a 2 mM DMPC solution with a 4.5 mM $TbCl_3$/2 mM DMPC solution. Each sample was sonicated for 1 h above 30 °C before use.

complex or a decrease in the amount of bound Tb^{3+} at the higher concentrations. NaCl facilitates this change in complexation, as shown in Fig. 2b. In order to distinguish these two possibilities, Tb^{3+} binding was studied by equilibrium dialysis. Dialysis experiments in absence of added NaCl were very erratic, with excessive loss of Tb^{3+} and DMPC to the dialysis membrane. Consequently, Table I contains results for only 0.010 M, 0.100 M and 1.00 M NaCl solutions. Three quantities were measured; the total Tb^{3+} concentrations in the DMPC chamber and the lipid-free chamber, and the Tb^{3+} emission intensity for the DMPC solutions. Because of the surface charge deposited on the zwitterionic DMPC by bound Tb^{3+}, the concentration of free Tb^{3+} in the solution depends on proximity to the charged DMPC surface. The measured Tb^{3+} concentration in the DMPC chamber is then given as

$$[Tb^{3+}]_L = [Tb^{3+}]_b + \langle [Tb^{3+}]_f \rangle \quad (1)$$

where the subscripts b and f stand for the bound and free concentrations and the brackets on the free concentration indicate a volume-weighted average. The form for this average is discussed in Appendix I, available upon request from the BBA Database. Using the results in Table I, and the methods outlined in Appendix I, the average free Tb^{3+} concentrations were found to be within 5% of the corresponding Tb^{3+} concentration in the lipid free chamber. Consequently, the volume weighted average concentration is treated as equal to the concentration in the DMPC-free chamber. The bound state molar emissions given in Table I were calculated from the measured Tb^{3+} emission intensity of the DMPC solution using these relations and a molar emission of 0.05 mM^{-1} for the aquo species. The molar emissions clearly indicate more than one bound state for Tb^{3+}.

Chrzeszczyk et al. [20] provide a convincing argument for a stoichiometry of two phosphate moieties per one bound trivalent lanthanide, based on limiting values for lanthanide induced shifts in the lipid ^{31}P-NMR band. Assuming this stoichiometry, the simplest model for Tb^{3+} binding yields

$$K_{app} = \frac{[TbS^{3+}]}{[S][Tb^{3+}]} = \frac{[Tb^{3+}]_b}{[Tb^{3+}]_i[DMPC]/2} \quad (2)$$

Here the surface is assumed to be composed of discrete sites, S, rather than discrete DMPC molecules. Subsequent analysis suggests this is approximately valid up to 15% site occupation. All three NaCl concentrations demonstrate decreasing K_{app} values with increasing bound cation. This is most likely a result of repulsion between free Tb^{3+} and the increasing surface charge density. The intrinsic binding coefficient depends on the Tb^{3+} concentration in the aqueous-membrane interface, which is related to the bulk concentration by a Boltzmann factor [5,6].

$$y_0 = \exp\{-FV_0/RT\} \quad (3)$$

$$K_0 = \frac{X_+}{(1-X_+)[Tb^{3+}]_i y_0^3} \quad (4)$$

TABLE I

Tb^{3+}-DMPC EQUILIBRIUM DIALYSIS; NaCl DEPENDENCE

$[Tb^{3+}]_L$, total Tb^{3+} concentration in the DMPC dialysis chamber. The quantity in parentheses is the standard deviation among three or four replicates. $[Tb^{3+}]_i$ Tb^{3+} concentration in the lipid-free chamber. K_{app}, apparent binding coefficient calculated from Eqns. 1–5. K_0, binding coefficient with Guoy-Chapman correction. V_0, surface potential associated with K_0. E_b, bound state molar emission $\times 10^{-3}$.

[NaCl] (M)	$[Tb^{3+}]_L$ (μM)	$[Tb^{3=}]_i$ (μM)	K_{app} (M^{-1})	K_0 (M^{-1})	V_0 (mV)	E_b (M^{-1})
0.010	26.9	11.2	1400	22000	24	112
	(0.8)	(0.3)				(11)
0.010	163	133	310	84000	49	113
	(14)	(16)				(12)
0.100	36.7	2.5	14000	101000	17	99
	(2.1)	(0.4)				(17)
0.100	266	171	610	82000	42	19
	(2)	(5)				(2)
1.00	34.2	3.8	8300	15000	5	38
	(2.5)	(1.0)				(4)
1.00	152	42	2900	21000	17	23
	(2)	(12)				(5)
			4700	54000		
			(120%)	(71%)		

where V_0 is the membrane surface potential in volts, X_+ is the mole fraction of sites occupied by Tb^{3+}, F is Faraday's constant, R is the gas constant in mks units and T is the temperature in Kelvin. The change from site concentration to mole fraction is a matter of convenience in the following treatment. The Boltzmann factor depends on the surface charge density, solution ionic strength and geometry of the charged membrane. In these experiments, small unilamellar vesicles (SUVs) were used to minimize the effects of light scattering at the high lipid concentrations required. Turbidimetric studies of these SUVs demonstrated the vesicle size to be strongly dependent on both the ionic strength and level of bound Tb^{3+}. The size also varied slowly with time. This uncertainty in vesicle size obviates the added effort required to treat the surface as a sphere. Here, the Boltzmann factor will be approximated by appling the Guoy-Chapman solution to the Poisson-Boltzmann equation, as done by Grasdalen et al. [5]. For these solutions, this is

$$\sigma = 2RT\epsilon\epsilon_0 \times 10^3 |[Tb^{3+}]_i (y_0^3 - 1) + [Na^+]_i (y_0 - 1) + [Cl^-]_i (y_0^{-1} - 1)| \quad (5)$$

$$\sigma = 3eX_+/2A \quad (6)$$

where σ is the surface charge density, ϵ_0 is the permittivity of free space in terms of $C^2 \cdot N \cdot m^{-2}$, e is the charge on an electron in coulombs (C), ϵ is the dielectric constant of water (76 at 30 °C), A is the surface area per DMPC assumed to be $7.0 \cdot 10^{-19}$ m^2, and the factor of 10^3 converts m^3 to liters. Given X_+ and the infinity concentrations, Eqn. 5 was solved iteratively using the Newton-Raphson approximation. Values for K_0 and V_0 are presented in Table I. The percent variation among the binding coefficients dropped from 120% for K_{app} to 71% for K_0, supporting the assumption that the double layer effect plays a substantial role in Tb^{3+} binding [5]. Agreement among the K_0 values is further improved if the infinity concentration of Tb^{3+} is adjusted with the mean activity coefficient at the solution ionic strength

$$K_0' = K_0/\gamma_\pm \quad (7)$$

The K_0' and $\gamma_\pm$ values are presented in Table II, with the latter estimated from experimental values for pure lanthanide trichloride solutions [23]. The standard deviation in K_0' is 59%, which is a rea-

TABLE II

Tb^{3+}-DMPC EQUILIBRIUM DIALYSIS; CONTRIBUTIONS FROM THE MEAN ACTIVITY COEFFICIENT AND CHLORIDE BINDING

K_0, binding coefficient with Guoy-Chapman correction. $\gamma_\pm$, mean activity coefficient estimated from lanthanide trichloride solutions [23]. K_-, chloride-DMPC binding coefficient. V_0, surface potential associated with K_0. K_0', Tb^{3+}-DMPC binding coefficient with contributions from the activity coefficient and chloride binding. Average K_0', the average value of K_0' for a given value of K_-; the values in parentheses are the relative deviations.

[NaCl] (M)	K_0 (M^{-1})	$\gamma_\pm$	K_- (M^{-1})	V_0 (mV)	K_0' (M^{-1})	Average K_0' (M^{-1})
0.010	22000	0.75	0	23.8	30000	
			0.01	23.6	29000	
			0.065	22.4	26000	
			0.1	21.7	24000	
0.010	84000	0.75	0	49.0	112000	
			0.01	48.7	106000	
			0.065	46.3	81000	
			0.1	45.1	70000	
0.100	101000	0.5	0	16.8	203000	
			0.01	16.2	190000	
			0.065	13.5	138000	
			0.1	11.9	116000	
0.100	82000	0.5	0	42.4	164000	
			0.01	41.1	143000	
			0.065	36.0	76000	
			0.1	33.5	59000	
1.00	15000	0.25	1	4.8	59000	
			0.01	3.6	52000	
			0.065	1.9	42000	
			0.1	−3.5	23000	
1.00	21000	0.25	0	16.9	85000	109000 (59%)
			0.01	15.3	70000	98000 (62%)
			0.065	8.7	33000	66000 (63%)
			0.1	5.9	24000	52000 (71%)

sonable uncertainty given the assumptions made and that the electrostatic corrections cover two decades in NaCl concentrations.

Grasdalen et al. [5] found it necessary to include weak chloride binding in their calculations of the surface potential. This is consistent with a recent study of anion binding to phosphatidylcholine in which Cl^- was found to have an intrinsic binding coefficient of less than 1 M^{-1} [10]. Assuming Cl^- binding involves individual choline moieties and is independent of Tb^{3+} binding

$$K_- = \frac{X_-}{(1-X_-)[Cl^-]_i y_0^{-1}} \tag{8}$$

$$\sigma = e(3X_+/2 - X_-)/A \tag{9}$$

where X_- is the mole fraction of DMPC with bound Cl^-. Table II contains values for K_0' obtained with the modified charge density (Eqn. 9) using 0.01 M^{-1}, 0.065 M^{-1} and 0.10 M^{-1} for K_-. Eqns. 4, 5, 6 and 9 were combined and solved by the Newton-Raphson method. Including Cl^- binding does not improve the agreement among the K_0' values, however, other evidence suggests anion binding to DMPC does play a role in the lipid phase state.

Table III shows the effect of electrolyte identity on Tb^{3+} binding. Perchlorate greatly enhances Tb^{3+} binding relative to chloride, while morpholinoethanesulfonate (Mes^-) greatly reduces binding. The former is consistent with microelectrophoretic measurements in which ClO_4^- was found to have an intrinsic binding coefficient of 220

TABLE III

Tb^{3+}-DMPC EQUILIBRIUM DIALYSIS: VARIOUS ELECTROLYTES

$[Tb^{3+}]_L$, total Tb^{3+} concentration in the DMPC dialysis chamber. The quantity in parentheses is the standard deviation among three or four replicates. $[Tb^{3+}]_i$, Tb^{3+} concentration in the lipid-free chamber. K_{app}, apparent binding coefficient calculated from Eqns. 1–5. E_b, bound state molar emission $\times 10^{-3}$.

0.100 M	$[Tb^{3+}]_L$ (μM)	$[Tb^{3+}]_i$ (μM)	K_{app} (M^{-1})	E_b (M^{-1})
$NaClO_4$	390	60	8100	20
	(13)	(12)	(960)	(2)
NaMes	274	266	30	585
	(19)	(15)	(65)	(80)
NaCl	266	171	610	19
	(2)	(5)	(26)	(2)
LiCl	263	202	320	35
	(26)	(19)	(130)	(6)
TMACl	260	196	350	81
	(14)	(4)	(70)	(6)

M^{-1} for phosphatidylcholine [10]. This is three orders of magnitude greater than the value for Cl^- estimated from the same studies [10]. Using 220 M^{-1}, in 0.1 M ClO_4^- the DMPC membrane has a net negative surface charge even with one third of the sites occupied by Tb^{3+}. The enhanced Tb^{3+} binding with this counterion is a consequence of this net negative charge. Mes^- is a very bulky ion which apparently has less affinity for DMPC than Cl^-. This is consistent with the lower affinity exhibited by SO_4^{2-} in the same electrophoretic measurements [10]. It is also likely the size of Mes^- reduces its screening efficiency in the double layer [24]. The cations Li^+ and tetramethylammonium (TMA^+) both reduce Tb^{3+} binding relative to solutions containing Na^+, although the effect is much less significant than that of the anions. No attempt was made to include monovalent cation binding in the surface charge density, however, competitive Na^+ binding may be partly responsible for the somewhat lower K_0 values at 1.0 M NaCl (Table I).

In both Tables I and III it is evident that the bound state molar emission, E_b, changes with the mole fraction of Tb^{3+} sites occupied, X_+. There is no similar correlation between E_b and the intrinsic binding coefficients. These observations suggest an ionotropic phase transition in the DMPC membrane which has virtually no effect on Tb^{3+} binding. Fig. 3 is a plot of E_b versus X_+ for the data in Fig. 2b. The values of E_b were calculated by combining Eqns. 2–9 and solving for X_+, X_- and y_0 iteratively. As a matter of caution in these calculations, several points in the titrations yield two solutions for y_0 which are close in value. Although only one is physically meaningful, the solution obtained is sensitive to the initial guess in the itteration. The symbols in Fig. 3 represent the E_b values with K_- set to 0.065 M^{-1} and the one-sided error bars locate the values of E_b and X_+ calculated with K_- set to zero; in most cases the symbols are larger than the these error bars.

Fig. 3 suggests the ionotropic transition is complete at 5–10% site occupation by Tb^{3+}. This is consistent with ultrasonic absorption studies in which Ca^{2+} was found to distort phosphati-

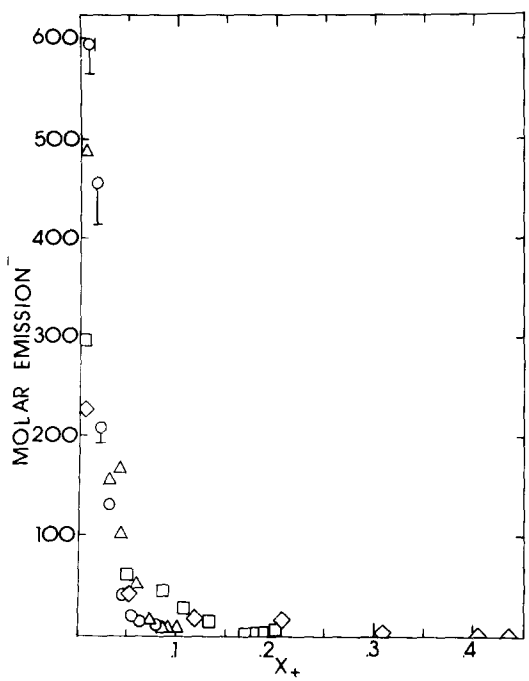

Fig. 3. Bound state molar emission (E_b) versus mole fraction of Tb^{3+} sites occupied (X_+). Values of E_b and X_+ were calculated using 66000 M^{-1} for K_0' and 0.065 M^{-1} for K_-: no added NaCl (circles), 0.010 M (triangles), 0.100 M (squares) and 1.00 M NaCl (diamonds). Using 109000 for K_0' and no Cl^- binding yielded values for E_b and X_+ which fall within the size of the symbols or are indicated by single-sided error bars.

dylcholine packing for several nanometers about the binding center [15]. Assuming a two state model for the lipid transition and that the Tb^{3+} molar emission reflects the change in phase state

$$K_t = \frac{X_2}{(1 - X_2)} \tag{10}$$

$$X_2 = (E_1 - E_b)/(E_1 - E_2) \tag{11}$$

where X_2 is the mole fraction of bound Tb^{3+} in the second state, which is assumed to be proportional to the fraction of DMPC in this state. K_t is an equilibrium coefficient for the lipid phase transition. E_1 is assumed to be 585 mM^{-1}, the value obtained from the MES^{1-} dialysis experiment, and E_2 is assumed to be the asymptotic value, 15 mM^{-1}. Recall, the molar emission for the aquo species is 0.05 mM^{-1} on the scale being used. In this model, the bound ions alter the activity of the DMPC molecules within the membrane. A virial expansion was chosen to represent this effect on the membrane activity:

$$-RT \ln K_t = \Delta G^0 + a_+ X_+ + a_- X_- + a_{+-} X_+ X_- + a_{++} X_+ X_+ \tag{12}$$

where ΔG^0 is the standard free energy change for the transition in absence of bound ions, a_+ and a_- are the surface chemical potentials for the bound ions and a_{+-} and a_{++} are second-order virial coefficients. The coefficients in this equation can be estimated by multiple linear regression using the values of X_+, X_- and E_b calculated previously. For a given NaCl concentration, a reasonably good fit is obtained using only the ΔG^0 and a_+ terms, although the parameter values vary systematically with NaCl concentration (Table IV). Regression of the combined data for the three NaCl concentrations was performed using all rational combinations of the terms in Eqn. 12. Two forms yielded similar average deviations among the experimental and regression values for E_b: a three-parameter fit in X_+ only and the full five-parameter fit. The residuals for these two regressions are shown in Fig. 4. The five-parameter fit (solid circles) clearly provides a better regression at low values of X_+ where most of the

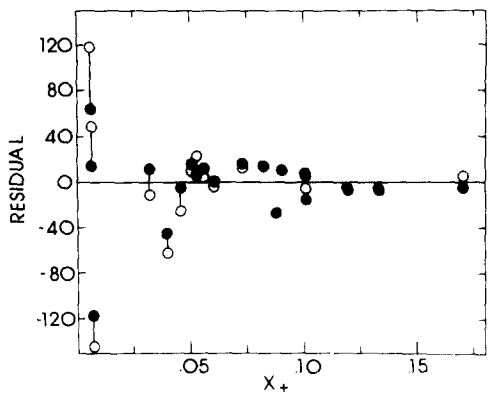

Fig. 4. Residuals from the three- and five-parameter regressions for the ionotropic transition. The filled circles are for the five-parameter fit using Eqn. 14 and the open circles are for the fit using only ΔG^0, a_+ and a_{++} as parameters. Vertical lines connect equivalent points in cases where the open and filled circles do not overlap. Regression was performed using data with X_+ less than or equal to 0.2. The data set for which there was no added Nacl was omitted; the intrinsic binding parameters seem to underestimate the amount of bound Tb^{3+} at very low ionic strength.

transition occurs. Since the ionotropic transition is virtually complete by 10% occupation, the five-parameter fit has been assumed to be most representative of the data because of the better agreement at low X_+. Consequently, Tb^{3+} and Cl^- are both directly involved in the ionotropic transition.

The infrared spectrum of the lipid demonstrates that Tb^{3+} binding does not change with the gel to liquid-crystal transition. Fig. 5 shows the phosphate stretch region of the infrared spectrum [25] for 200 mM DMPC and the same with 40 mM $TbCl_3$. The multiple overlayed spectra are for several temperatures above and below and gel to liquid-crystal transition temperature. Tb^{3+} binding is signalled by the shoulder at roughly 1120 cm^{-1}. The divalent anionic lipid, cardiolipin, demonstrates a similar but more pronounced shift in the phosphate region upon complexation with Tb^{3+} *. This shoulder in the Tb^{3+}-DMPC spectrum remains intact throughout the thermotropic transition, indicating that Tb^{3+} binding is not dependent on the DMPC phase state.

* Fig. 7. Phosphate stretch region of the cardiolipin infrared spectrum. Available upon request, see footnote on p. 53.

TABLE IV

DMPC IONOTROPIC PHASE TRANSITION: THERMODYNAMIC PARAMETERS

[NaCl], molar concentration of NaCl for the data set from Fig. 2b used in the regression with Eqn. 15; 'all' refers to the three data sets combined. Only data with X_+ less than or equal to 0.20 were used. ΔG^0, the change in free energy for the ionotropic transition in absence of bound ions; kilojoules per mole DMPC. a_+, units of kilojoules per mole fraction of occupied Tb^{3+} sites. a_{++}, units of kilojoules per (mole fraction of occupied Tb^{3+} sites) [2]. a_-, units of kilojoules per mole fraction of DMPC having bound Cl^-; K_- was assumed to be 0.065 M^{-1}. a_{+-}, units of kilojoules per mole fraction of occupied Tb^{3+} sites per mole fraction of DMPC having bound Cl^-. Average deviation, the route-mean-square relative deviation in the molar emission values calculated from the regression equation.

[NaCl]	ΔG^0	a_+	a_{++}	a_-	a_{+-}	Av. dev.
1.00	−2.9	−36	–	–	–	47%
0.100	−0.54	−66	–	–	–	11%
0.010	3.6	−160	–	–	–	8%
All	−1.5	−61	–	–	–	163%
All	1.9	−168	5630	–	–	35%
All	2.3	−174	622	−21	–	37%
All	2.4	−165	479	−41	360	34%

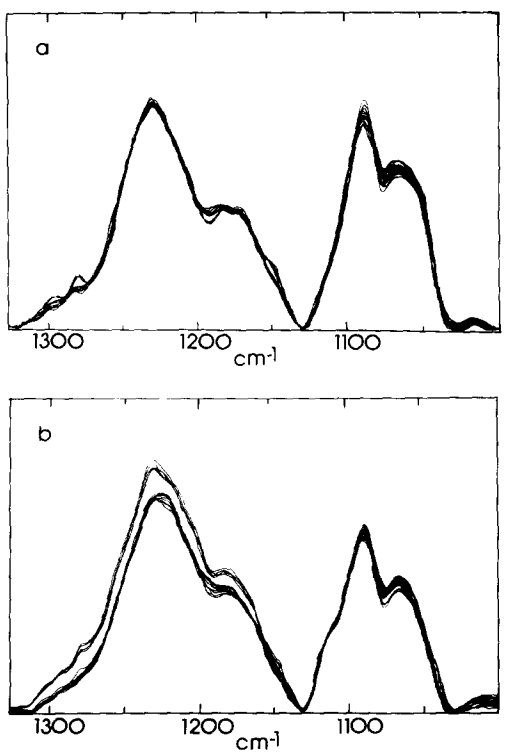

Fig. 5. Phosphate stretch region of the DMPC infrared absorption spectrum. Overlayed spectra for discrete temperatures from 18° C to 35° C: (a) 0.2 M DMPC; (b) 0.2 M DMPC with 40 mM $TbCl_3$. There are several absorption bands in this region: P = O stretch at 1250 and 1085 cm^{-1}, C-O-C stretch at 1170 and 1070 cm^{-1} and a shoulder at 1120 cm^{-1} in the Tb^{3+}-DMPC spectrum which appears to be part of the 1085 cm^{-1} P = O band shifted to higher energy. Except for the last, the assignments are those provided by Casal and Mantsch [25].

The methylene stretch region [25] is better resolved than the phosphate bands and more clearly reflects the thermotropic transition (Fig. 6). Fig. 6c is a plot of the methylene asymmetric stretch frequency as a function of temperature. As expected from calorimetric studies, bound lanthanide shifts the transition to higher temperatures and reduces the cooperativity [12]. Both the methylene asymmetric stretch (2920 cm^{-1}) and the symmetric stretch (2850 cm^{-1}) have much narrower band widths for Tb^{3+}-DMPC (Fig. 6a) than for DMPC alone (Fig. 6b) indicating a more rigid phase for the former both above and below the transition. Thus, the perturbation introduced by bound Tb^{3+} is not restricted to the interfacial region but extends throughout the acyl chains.

Discussion

This work suggests there are two types of cooperative interaction between cations and anions bound to phosphatidylcholine membranes. One has to do with the net charge deposited by the bound ions and its effect on the ion concentrations at the membrane-aqueous interface. Previous studies demonstrated this first cooperativity [5,11], and related work has dealt with cation [6,14,15,20] and anion [10] binding individually.

The second form of cooperativity deals with an ionotropic transition in the DMPC membrane. This transition was manifested as a change in

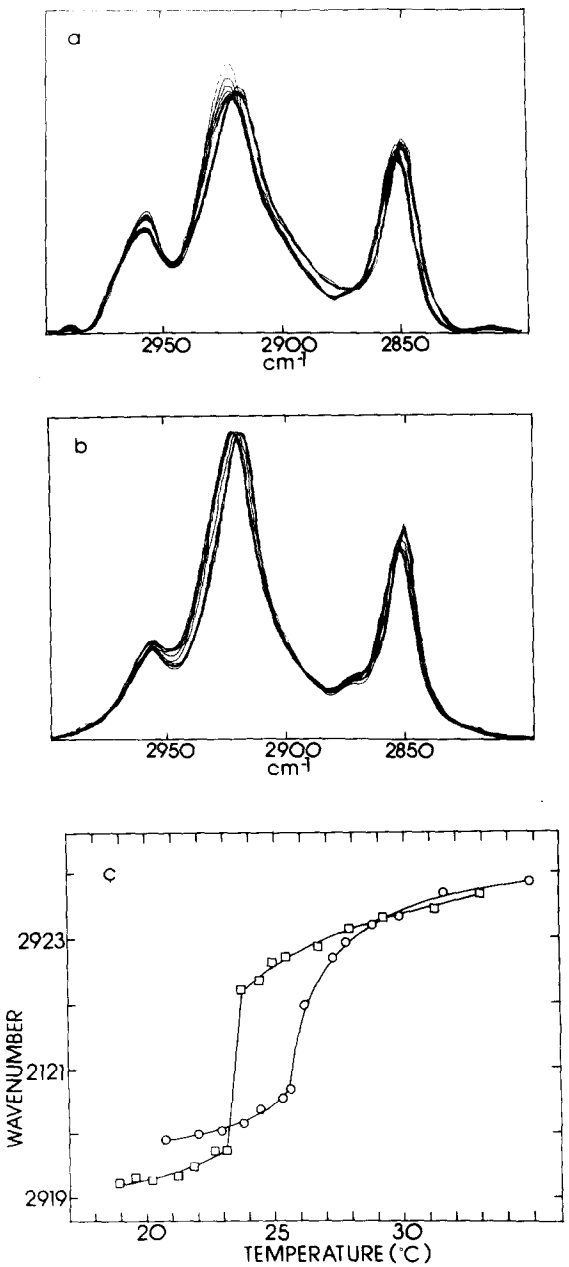

Fig. 6. Methyl and methylene stretch region of the DMPC infrared spectrum. Overlayed spectra for discrete temperatures from 18 °C to 35 °C: (a) 0.2 M DMPC; (b) 0.2 M DMPC with 40 mM $TbCl_3$. Methyl stretch bands [25] are located at 2960 and 2870 cm^{-1}; the methylene bands [25] are at 2920 and 2850 cm^{-1}. (c) The position of the 2920 cm^{-1} methylene band is particularly sensitive to the DMPC gel to liquid-crystal transition. This is evident from the overlayed spectra, but is shown more clearly in this plot of band position versus temperature: circles represent the Tb^{3+}-DMPC spectrum and squares are for DMPC alone.

bound Tb^{3+} emission efficiency, narrower infrared bands, and a shift to higher temperatures for the gel to liquid-crystal transition. The effect of bound Tb^{3+} on the thermotropic transition is consistent with calorimetric studies of La^{3+}-dipalmitoylphosphatidylcholine (DPPC) complexes [12]. Narrowing of the infrared absorption bands upon Tb^{3+} binding suggests a transition to a more rigid lipid phase. Ca^{2+} binding to phosphatidylcholine has also been shown to result in a more rigid lipid membrane using ultrasonic absorption [15] and fluorescence depolarization [14] measurements. Other evidence that cation binding alters the phosphatidylcholine phase state has been provided by both X-ray diffraction studies [13] and ^2H-NMR [6]. Specific anion binding to phosphatidylcholine has been demonstrated by microelectrophoresis [10] and osmotic pressure measurements [11]. In the work presented here it was necessary to invoke anion binding as well as Tb^{3+} binding in modeling the ionotropic transition of DMPC.

Discussion of either cooperativity requires knowledge of the levels of bound ions. There has been some disagreement, however, over the particular binding model to be used in lanthanide (III)-phosphatidylcholine studies. Grasdalen et al. [5], found their data to be consistent with a single bound state for Tb^{3+} and a Guoy-Chapmann form for the effect of the deposited surface charge, if weak chloride binding was included. Chrzeszczyk, et al. [20], found Pr^{3+}-DPPC complexation to be best represented by two bound states for the cation and a parametric correction for ionic strength and deposited charge, with neglect of anion binding. The intrinsic binding coefficient at low levels of Pr^{3+} was estimated as 2 M^{-1} and at higher levels of bound Pr^{3+} the surface was assumed to undergo a transition to a 'relaxed' state with a binding coefficient of 3000 M^{-1}. The equilibrium dialysis results presented in Tables I and II are in conflict with these binding parameters, regardless of the method for treating electrostatic contributions. Although the Tb^{3+} emission reflects two bound states, there is no correlation between the relative population of these states and the intrinsic binding coefficient. There is at least one reasonable explanation for the bimodal binding observed in the Pr^{3+} studies that has to do with the counter

ions involved: NO_3^- and dimethylphosphate (DMP^-). Pr^{3+} was added to DPPC as the NO_3^- salt and DMP^- was present as an indicator of free Pr^{3+} activity [20]. Throughout the Pr^{3+} titration of DPPC the ratio of NO_3^- to DMP^- increased. Tatulian [10] demonstrated that NO_3^- has a greater affinity for phosphatidylcholine than Cl^- and SO_4^{2-}. Although there is, as yet, no direct evidence, it seems likely that DMP^- has a lower affinity for phosphatidylcholine than does NO_3^-. Given the very drastic effect the anion can have on the apparent binding of the lanthanide, e.g. Cl^- versus Mes^- for Tb^{3+}(Table III), the varying anion composition in the Pr^{3+} experiments could be the source of the bimodal binding.

These Tb^{3+} studies are well represented by a model most similar to that of Grasdalen et al. [5]. The differences are in the Tb^{3+}-DMPC stoichiometry and how it is represented in the equilibrium expressions. Chrzeszczyk et al. [20] provide a good argument for two DMPC molecules per binding site, which has been assumed here. Grasdalen et al. [5] assumed three or one lipid molecules per site. Here, the membrane surface is treated as being composed of discrete binding sites, rather than discrete lipid molecules. Although this assumption should break down at high levels of bound Tb^{3+}, the intrinsic binding coefficients calculated from the dialysis experiments show no evidence of this occurring for up to 15% site occupation.

Although there is no clear trend, the values for the binding coefficients at 1.0 M NaCl appear lower as a group (Table II). It is possible the surface potential is more positive than calculated as a result of weak Na^+ binding [9], which is less pronounced at lower concentrations. Na^+ binding is suggested by the lower apparent Tb^{3+} binding in the presence of LiCl and TMACl (Table III). A second possibility for the lower intrinsic binding at 1.0 M NaCl may be that the mean activity coefficient for Tb^{3+} is overestimated by roughly a factor of two. This is likely given that the activity coefficients were estimated from those of pure lanthanide trichlorides at equivalent ionic strengths. The dialysis solutions in question had 1.0 M NaCl and less than 0.001 M $TbCl_3$, which represents a difficult mixed electrolyte problem to deal with experimentally. It seems equally likely that the deviation about the average intrinsic binding coefficient may simply be the uncertainty in these dialysis experiments, given the 1.0 M NaCl values agree well with at least one of the 0.010 M NaCl experiments (Table II). Additional work with this system will be required to resolve this question.

As part of the dialysis experiments, the bound state Tb^{3+} molar emission was determined. There are clearly at least two bound states for Tb^{3+} which are related by a phase transition in the DMPC membrane. This is shown more clearly in Fig. 3 where the molar emission drops precipitously with the mole fraction of membrane sites occupied by Tb^{3+}. The transition is nearly complete at roughly 10% occupation. A virial expansion of the change in free energy for the DMPC transition was used to account for the role of bound ions. The equilibrium expression for the ionotropic transition required first-order terms for bound Tb^{3+} and Cl^- as well as second-order Tb^{3+}-Tb^{3+} and Tb^{3+}-Cl^- terms for a reasonable regression of the combined Tb^{3+} emission studies in 0.010 M, 0.100 M and 1.00 M NaCl. The particular thermodynamic parameters obtained from this regression are not definite since it was necessary to assume an intrinsic binding coefficient for Cl^-. Grasdalen et al. [5] found 0.065 M^{-1} to give the best regression for their Tb^{3+} binding studies, which agrees with microelectrophoresis experiments [10] that indicate that it is less than 1 M^{-1}. For the multiple regression of the emission studies, 0.065 M^{-1} was assumed. The change in free energy for the transition is small (2–3 kJ/mol DMPC) and unfavorable in absence of bound ion. The first-order terms in the expansion represent favorable free energy contributions with Tb^{3+} binding being more significant, as expected. The second-order terms are necessary for a good fit to the data and represent unfavorable contributions to the transition free energy change. This is reasonable given that these emission studies and ultrasonic absorption studies [15] suggest that cation binding distorts the phase state of several local phosphatidylcholine molecules. Fig. 3 indicates the ionotropic transition is essentially complete at one Tb^{3+} bound for every 20–40 DMPC. This corresponds to roughly three nearest-neighbor layers if hexagonal packing of the DMPC is assumed.

Coalescence of two or more of these binding-site domains will likely result in fewer perturbed DMPC molecules per bound ion. Although there were several approximations, this argument is physically reasonable and consistent with the observations.

As demonstrated in previous studies, the gel to liquid-crystal thermotropic transition for phosphatidylcholine is perturbed toward higher temperatures as a result of cation binding [6,12]. Interestingly, anion binding appears to lower the transition temperature, although the evidence is not definitive [10]. The DMPC infrared spectrum also demonstrates a cation-induced shift of the transition to higher temperatures. The transition temperature increases from 23.6°C for pure DMPC to roughly 26°C under conditions where only a small fraction of lipid is bound to Tb^{3+}. The phosphate region of the spectrum indicates that the amount of bound Tb^{3+} remains constant throughout the transition. In the methylene region of the spectrum, the bands are narrower at all temperatures for the Tb^{3+}-DMPC sample, suggesting a more rigid state for both the gel and liquid-crystal phases of the lipid. The narrow bands with the Tb^{3+} complex give no evidence of phase separation of the complex from DMPC. This suggests an even dispersion of Tb^{3+} over the membrane surface, which was assumed in the surface charge density calculations. The sensitivity of the infrared spectrum to low levels of bound ion will prove useful in relating the Tb^{3+} emission studies to less tractable but biochemically more relevant ions.

The work presented here demonstrates the sensitivity of Tb^{3+} emission to subtle changes in its environment and gives evidence of cation and anion cooperativity in altering phosphatidylcholine phase behavior. This observation is particularly significant given three facts: phosphatidylcholine is a major lipid component in nearly all biological membranes; most membranes have a net negative surface charge; and the affinity of, say, Ca^{2+} is roughly the same for all phospholipids once a correction is made for the surface potential [5–9]. Thus, under most biological conditions, ion binding to phosphatidylcholine is likely to be as significant as that to any other phospholipid.

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