Biochimica et Biophysica Acta, 413 (1975) 341–353

BBA 77141

DIFFERENCES IN THE INTERACTION OF INORGANIC AND ORGANIC (HYDROPHOBIC) CATIONS WITH PHOSPHATIDYLSERINE MEMBRANES

H. HAUSER*, M. C. PHILLIPS and M. D. BARRATT

Biosciences Division, Unilever Research Laboratory Colworth/Welwyn, The Frythe, Welwyn, Herts. (U.K.)

(Received June 4th, 1975)

SUMMARY

The interaction of phosphatidylserine dispersions with "hydrophobic", organic cations (acetylcholine, tetraethylammonium ion) is compared with that of simple inorganic cations (Na^+, Ca^{2+}); differences in the hydration properties of the two classes of ions exist in the bulk phase as evident from spin-lattice relaxation time T_1 measurements. It is shown that the reaction products (cation-phospholipid) differ markedly in their physicochemical behaviour. With increasing concentration both classes of ions reduce the ζ-potential of phosphatidylserine surfaces, the monovalent inorganic cations being only slightly more effective than the hydrophobic cations. Inorganic cations cause precipitation of the lipid once the surface charge of the bilayer is reduced to a certain threshold value. This is not the case with the organic cations. The difference is probably associated with the different hydration properties of the resulting complexes. Thus binding of Ca^{2+} causes displacement of water of hydration and formation of an anhydrous, hydrophobic calcium-phosphatidylserine complex which is insoluble in water, whereas the product of binding of the organic cations is hydrated, hydrophilic and water soluble. The above findings are consistent with NMR results which show that the phosphodiester group is involved in the binding of both classes of cations as well as being the site of the primary hydration shell. Besides affecting interbilayer membrane interactions such as those involved in cell adhesion and membrane fusion, the binding of both classes of cation can affect the molecular packing within a bilayer.

INTRODUCTION

Ion binding and ion exchange processes at the surface of biological membranes are implicated in many physiological functions, e.g. nerve excitability. The phospholipid bilayer, which is an integral part of biological membranes [1], is likely to provide non-specific ion binding sites. Hence the binding of ions such as Na^+ and Ca^{2+} to

* Present address: Eidgenössische Technische Hochscule Zürich, Laboratorium für Biochemie, 8006 Zürich, Universitätstrasse 16, Schweiz. Reprint requests should be sent to this address.

phospholipid monolayers and bilayers has been widely studied (e.g. refs 2–5). However, organic ions such as acetylcholine are also associated with membranes in vivo, but the interaction of this type of ion has hardly been investigated. Here we compare the binding to phosphatidylserine of some hydrophobic, organic cations (acetylcholine and tetraethylammonium) and some simple inorganic cations (Na^+ and Ca^{2+}). We show that there are significant differences in the behaviour of the two types of system.

EXPERIMENTAL

(1) *Materials*

Ox brain phosphatidylserine was purchased from Lipid Products, South Nutfield, Surrey, U.K.; it was checked by thin-layer chromatography and where necessary purified by standard methods [6]. AnalaR grade NaCl, KCl, $CaCl_2$ and $MnCl_2$ were roasted at 500 °C before use. Tetramethyl- and tetraethylammonium chloride, tetramethylammonium bromide and acetylcholine chloride were obtained from B.D.H., Poole, U.K.; these materials were used without further purification. ^2H_2O was approx. 99.7 % (from Prochem Ltd, Croydon, U.K.). All other chemicals were AnalaR grade. The water used was distilled, deionized, distilled from alkaline permanganate under N_2 and redistilled in an all-glass apparatus.

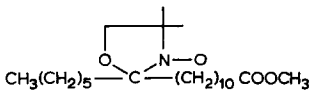
Sample preparation. Unsonicated phosphatidylserine dispersions for measuring electrophoretic mobilities were prepared as described in ref. 2. The preparation of sonicated phospholipid dispersions has been described before [7, 8]. Concentrated unsonicated phospholipid dispersions used for ^{31}P and ^2H wide-line NMR experiments were prepared in a tube containing a constriction [9]. The phospholipid and water or ^2H_2O were weighed in and thoroughly homogenized by centrifuging the mixture repeatedly backwards and forwards through the constriction. Dry weight determination of the samples showed the water content to be accurate to within ± 5 %. Unless otherwise stated the samples prepared for wide-line NMR experiments contained lipid and salt in equimolar concentrations. The lipid and salt were vacuum dried before use. Samples were prepared under N_2 at 20–25 °C.

(2) *Methods*

Surface chemistry. The procedures used to measure simultaneously surface pressure (π) and surface potential (ΔV) as a function of surface area/molecule at 20 ± 1 °C have been described before [10]. Electrophoretic mobilities were determined at 25 ± 0.5 °C in a cylindrical electrophoresis cell as described in ref. 2.

Nuclear magnetic resonance (NMR). ^1H spin-lattice relaxation time (T_1) measurements were obtained with a Brucker 270 MHz spectrometer using pulse Fourier transform techniques based on a 180-τ-90° pulse sequence. ^{31}P and ^2H wide-line experiments were carried out on a Varian Associates wide-line NMR spectrometer (12″ electro-magnet) operating at 8.13 MHz. A Northern Scientific NS 544 time averaging computer was attached to the spectrometer in order to enhance the signal-to-noise ratio [9]. Linewidths W and quadrupole splittings $\Delta\nu$ were measured from first-derivative presentation of the spectra as described elsewhere [9, 11]; the errors in $\Delta\nu$ are ± 50Hz (maximum) and ± 10 % in W. All NMR measurements were carried out at 25 ± 1 °C.

Certain samples were investigated by X-ray diffraction [9] and spin-probe methods [12]. The order parameter (S_3), as defined by Seelig [13], was determined for the spin-label incorporated in unsonicated dispersions of phosphatidylserine at a molar ratio of lipid/probe of 130/1.



Scheme 1.

RESULTS

Fig. 1 shows how the electrophoretic mobility of ox brain phosphatidylserine particles (liposomes) changes as a function of the concentration of various inorganic and organic cations. With inorganic cations aggregation and fusion of phosphatidylserine bilayers commenced at a certain concentration; this precipitation led to an increase in absorbance (see inset to Fig. 1). As pointed out before [2] there is a good correlation between the concentration of these cations required to reduce the electrophoretic mobility to $-1\ \mu s^{-1} \cdot V^{-1} \cdot cm$ and that required to increase the absorbance at 520 nm of sonicated phosphatidylserine dispersions [14] to an arbitrary value of 2. In contrast to the inorganic ions, addition of tetramethylammonium, tetraethylammonium and acetylcholine chlorides to phosphatidylserine dispersions did not give rise to turbidity and precipitation despite the fact that their effect on the

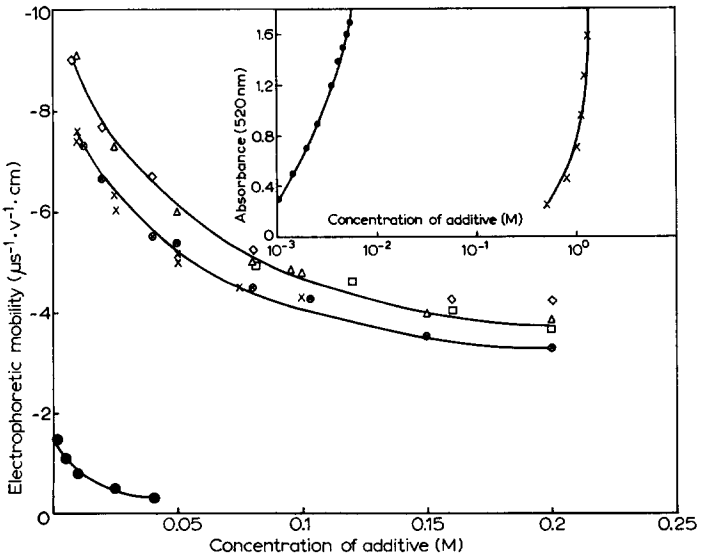


Fig. 1. Electrophoretic mobility in μs^{-1} per unit electric field strength ($V \cdot cm^{-1}$) of unsonicated phosphatidylserine particles as a function of ion concentration. ◇, tetramethylammonium chloride; □, tetraethylammonium chloride; △, acetylcholine chloride; ×, NaCl; ⊗, KCl; ●, $CaCl_2$. For experimental details see ref. 2. Inset: absorbance at 520 nm of sonicated phosphatidylserine vesicles [14] as a function of ion concentration. ●, $CaCl_2$; X, NaCl or KCl.

electrophoretic mobility and thus the ζ-potential was similar to that of the inorganic cations. In agreement with this observation that the inorganic and organic monovalent cations have similar effects on the surface charge, the surface potentials of phosphatidylserine monolayers spread on substrates containing these ions are the same within experimental error (Table I).

TABLE I

SURFACE PRESSURE AND SURFACE POTENTIAL OF MONOLAYERS OF OX BRAIN PHOSPHATIDYLSERINE AT 70 Å^2/MOLECULE (EQUIVALENT TO THE PACKING IN A LIQUID-CRYSTALLINE BILAYER) ON SUBSTRATES (pH 6) CONTAINING VARIOUS SALTS

Substrate	$\pi(\pm 1$ mN m$^{-1})$	ΔV (± 10 mV)
Distilled water	10	165
0.1 M acetylcholine chloride	25	250
0.1 M tetraethylammonium chloride	31	235
0.1 M NaCl	18	255
10^{-3} M $CaCl_2$	20	365

The properties of the water of hydration of the cations have been investigated by using pulsed NMR to determine spin-lattice relaxation times (T_1) [15, 16]. Fig. 2 demonstrates the different effects of inorganic and organic ions on water structure The relative deuteron relaxation rates $(1/T_1)_{rel}$ of ^2H_2O are shown as a function of salt concentration.

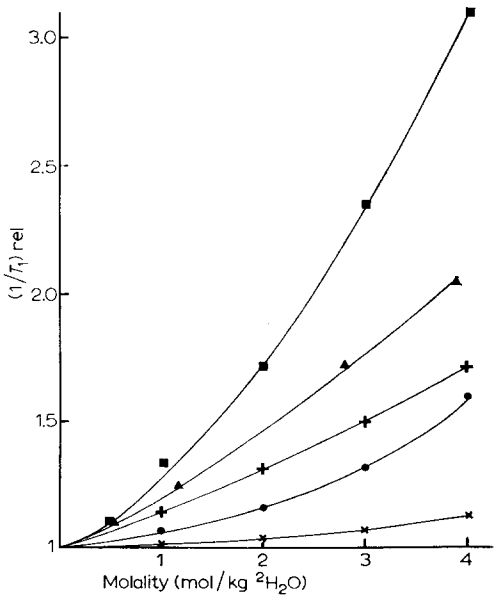


Fig. 2. Relative relaxation rates $(1/T_1)_{rel}$ of ^2H_2O at 25 °C as a function of salt concentration (mol/kg ^2H_2O); ■, tetraethylammonium bromide [16]; ▲, acetylcholine chloride; ●, tetramethylammonium bromide [16]; +, $CaCl_2$ [15, 17]; ×, NaBr [17].

$$(1/T_1)_{rel} = (1/T_1)/(1/T_{1(0)}) \quad (1)$$

where $T_{1(0)}$ and T_1 are the deuteron relaxation times of pure ^2H_2O and of ^2H_2O in the presence of salt, respectively. The increase in $(1/T_1)_{rel}$ is largest with $(C_2H_5)_4NBr$ and the effect is in the order tetraethylammonium bromide > acetylcholine chloride > $CaBr_2 \approx CaCl_2$ > tetramethylammonium bromide > NaBr. If the ionic effect of a 1 : 1 electrolyte is considered to be represented by NaBr or tetramethylammonium bromide the increase in $(1/T_1)_{rel}$ observed with tetraethylammonium bromide or acetylcholine chloride may then be attributed to the effect of the additional alkyl groups. Such a hydrophobic hydration effect is assumed to arise from clathrate formation around the hydrophobic residues [19]. The increase in $(1/T_1)_{rel}$ of Ca^{2+} compared to that of Na^+ is probably due to the higher charge density of Ca^{2+} and assumed to be an electrostrictive effect [18].

TABLE II

DISTANCE RATIOS r_{ij} DERIVED FROM LINE BROADENING AND T_1 MEASUREMENTS WITH SONICATED AQUEOUS DISPERSIONS OF EGG LECITHIN IN THE PRESENCE OF $GdCl_3$

r_{ij} is the ratio of the distance of nucleus i from Gd^{3+} and the distance Gd^{3+}-$OPCH_2$ protons.

Nucleus i	Line broadening (r_{ij})	T_1 measurement (r_{ij})
^{31}P	0.65±0.15	–
$POCH_2$ (choline)	1.00	1.00
CH_2N	1.08±0.05	1.12±0.04
$N(CH_3)_3$	1.20±0.06	1.24±0.04
CH_2OP (glycerol)	–	1.03±0.05

In a previous study of ion binding to phosphatidylcholine [20], distance ratios (Table II) were obtained by monitoring the effect of increasing $GdCl_3$ concentrations on the linewidth at half height of the ^{31}P resonance and three ^1H resonances from the phosphorylcholine groups of a sonicated dilauroyl phosphatidylcholine dispersion. Here we have investigated the effects of salts on the motions of the polar group of phosphatidylserine by determining the ^{31}P linewidths of wide-line NMR spectra recorded from ox brain phosphatidylserine/water mixtures in the presence and absence of salts. Table III summarises the effects of the salts on the linewidths obtained from both phosphatidylserine/water and phosphatidylcholine/water mixtures; only with the samples containing tetraethylammonium ion (9 mol water/mol lipid) and Ca^{2+} did the linewidth depart significantly from the average, indicating that the motions of the phosphorus atoms are severely restricted (for $CaCl_2$ the restriction is such that the spectrum is too broad to be detected). When tetraethylammonium ion was added to samples containing more water (20 mol water/mol lipid) the ^{31}P linewidth was typical of the phospholipid in the absence of salt.

Table IV summarises the characteristics of the ^2H spectra of ^2H_2O added to phosphatidylserine in the absence and presence of ions. Only singlet spectra were obtained when Ca^{2+} was added whereas in the presence of Na^+ or tetraethylammonium ion singlets as well as doublets were observed.

TABLE III

LINEWIDTHS W(Hz) FOR THE ^{31}P WIDELINE RESONANCE OF PHOSPHOLIPID-WATER MIXTURES AT 24 °C

n, molecules of water added per phospholipid molecule.

Phospholipid	Salt*	n	W(Hz)
Egg lecithin (unsonicated)	none	21	420 (51.7 ppm)
	none	37	470
	NaCl	18	280
	tetraethylammonium chloride	9	440
	$CaCl_2$	38	360
Egg lecithin (sonicated)**	none	$\approx$ 200	36 (1 ppm)
	$CaCl_2$	$\approx$ 200	36
Monosodium salt of ox brain phosphatidylserine	none	17	280
	none	39	310
	NaCl	20	230
	tetraethylammonium chloride***	9	2340
	tetraethylammonium chloride	21	350
	$CaCl_2$***	20	signal too broad to be detected

* Salt and lipid were present in equimolar concentrations.

** High-resolution NMR spectra were obtained from 2 % (w/v) dispersions of egg lecithin in water sonicated to single bilayer vesicles [8].

*** The phospholipid-water mixture was first homogenized as described in Experimental, the salt was added and the whole was then rehomogenized.

TABLE IV

DOUBLET SPLITTINGS ($\Delta\nu$) AND SINGLET LINEWIDTHS (W) FROM ^2H SPECTRA OF ^2H_2O/SALT/PHOSPHATIDYLSERINE MIXTURES AT 24 °C

In all samples except those marked** salt and lipid were mixed in equimolar proportions. n, mol ^2H_2O per mol phospholipid and S denotes a sharp singlet of width < 40 Hz limited by the spectrometer modulation conditions. $\Delta\nu$ values in the absence of salt are taken from ref. 9.

Phospholipid	Salt	n	$\Delta\nu$ (kHz)	W(Hz)
Monosodium salt of ox brain phosphatidylserine	none	9	2.00	750*
	none	21	0.64	350*
	NaCl	20	1.67	S
	tetraethylammonium chloride	21	2.41	S
	$CaCl_2$	8	–	S
	$CaCl_2$	20	–	S
	$CaCl_2$**	10	–	65
	$CaCl_2$**	21	–	S
	NaCl**	18	1.56	53

* For a discussion of the origin of this line see ref. 9.

** In these preparations salt was added to 1–5 % (w/v) sonicated dispersions of lipid until precipitation occurred (cf. inset of Fig. 1). The insoluble $CaCl_2$ · phosphatidylserine precipitate was first washed with ^2H_2O, and freeze-dried, then the required amount of ^2H_2O was added and the mixture homogenized.

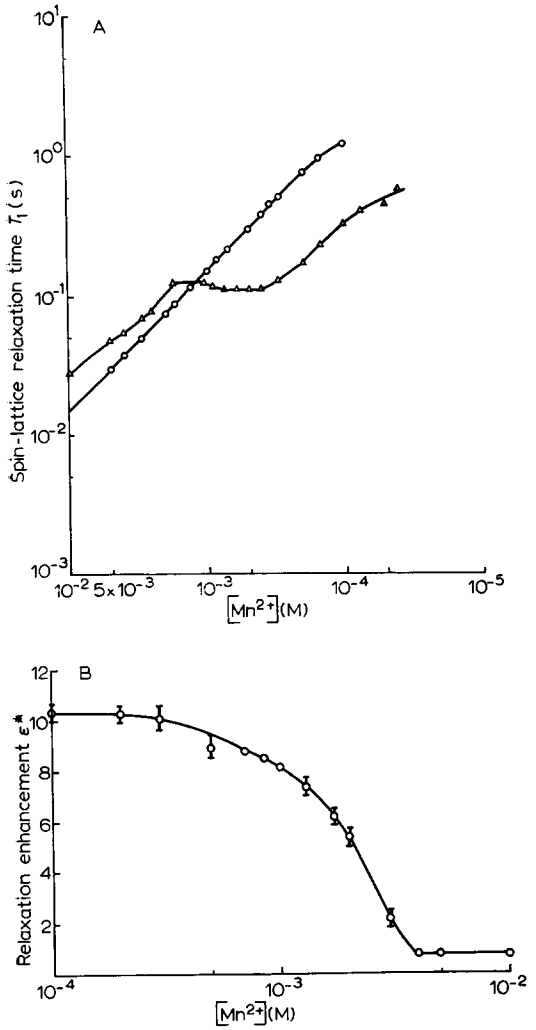


Fig. 3.(a) Proton spin-lattice relaxation time (T_1) of water (0) and of a 10^{-3} M sonicated dispersion of crude sodium phosphatidylserine in 0.05 M Tris · HCl, pH 7.4 (△) as a function of Mn^{2+} concentration at 24 °C. The phospholipid contained up to 10 % impurities (mainly lyso compounds) as detected by thin-layer chromatography analysis. Since the polar groups of the impurities are similar to that of phosphatidylserine the conclusions derived from those experiments are not significantly affected by this level of contamination. (b) Relaxation enhancement ε^* defined and calculated according to Eqn 1 as a function of Mn^{2+} concentration. Experimental conditions as described under a. The bars give the spread of 2–3 measurements.

Fig. 3a gives the spin-lattice relaxation time T_1 of pure water and of water containing 10^{-3} M sonicated phosphatidylserine [14] as a function of the Mn^{2+} concentration. Sonicated dispersions gave more reproducible results than unsonicated dispersions. At $[Mn^{2+}] \leqslant 10^{-3}$ M, T_1 is significantly shorter in the presence of phosphatidylserine. At $[Mn^{2+}] \geqslant 10^{-3}$ M, T_1 approaches the values of Mn^{2+} solutions in the absence of phosphatidylserine and eventually becomes even longer.

Fig. 3b is a plot of the relaxation enhancement ε^* [21, 22] as a function of Mn^{2+} concentration. The empirical relaxation enhancement ε^* was calculated from T_1 measurements according to Eqn 2

$$\varepsilon^* = \frac{(1/T_1^*)-(1/T_1^*{}_{(0)})}{(1/T_1)-(1/T_{1(0)})} \quad (2)$$

where $1/T_{1(0)}$ is the spin-lattice relaxation time in the absence of Mn^{2+} and the asterisk indicates the presence of 10^{-3} M sonicated phosphatidylserine. At $[Mn^{2+}] \leqslant 10^{-3}$ M ε^* values are of the order of 10 while at $[Mn^{2+}] \geqslant 10^{-3}$ M ε^* decreases rapidly towards a final value of less than 1.

DISCUSSION

Hydration and ion binding to phosphatidylserine

Earlier studies [20] of the effect (corrected for any diamagnetic effects) of Gd^{3+} on linewidth and T_1 of the phosphatidylcholine polar group resonances showed that the phosphate is nearer to the metal than any other group (Table II) indicating that the phosphodiester group is the metal binding site in phosphatidylcholine. Consistent with this the ^{31}P wide-line NMR experiments of Ca^{2+} · phosphatidylserine complexes show that the phosphate group is involved in the formation of the metal complex (Table III). In contrast to Ca^{2+}, tetraethylammonium ions at water contents $(n) \gtrsim 20$ mol per mol of lipid do not cause line broadening in the ^{31}P wide-line spectrum of phosphatidylserine (Table III). This result together with the observation of a singlet and doublet in the ^2H spectrum of tetraethylammonium ion/^2H_2O/phosphatidylserine mixtures (Table IV) suggests that the interaction of tetraethylammonium ion produces hydrated ion-lipid complexes in which the molecular motion is not changed significantly compared to phosphatidylserine in the absence of ions.

In general, ^2H spectra of ^2H_2O contain doublets if the motion of the ^2H-O bond averaged over a characteristic period of time is anisotropic, i.e. if the electric field gradient at the deuterium nucleus is not averaged out by rapid tumbling (for a more detailed discussion see ref 9 and 11). The doublet splitting is a measure of the degree of anisotropy of the motions of the ^2H_2O molecules and thus allows conclusions about the mode of ^2H_2O-phospholipid interactions [9]. In a two-phase phospholipid-^2H_2O system consisting of phospholipid bilayers at maximum hydration and excess ^2H_2O a doublet superimposed on a singlet is observed. The former originates from ^2H_2O molecules hydrating the phospholipid and the latter from isotropically tumbling water molecules which cannot exchange rapidly with water molecules in the hydration shells of the phospholipid [9, 11]. The ^2H spectra of ^2H_2O/phosphatidylserine mixtures both in the absence and in the presence of tetraethylammonium ion consist of a doublet and a singlet indicating that besides water of hydration free, isotropically tumbling, water is present. Inspection under the light microscope of these samples confirmed the presence of a two-phase system consisting of a lipid and an aqueous phase. Contrary to the tetraethylammonium ion-containing mixture, the ^2H-spectrum of ^2H_2O/phosphatidylserine plus $CaCl_2$ consists only of a singlet, indicating that only isotropically tumbling water is present. Low angle X-ray diffraction studies of Ca^{2+} · phosphatidylserine complexes showed that the multilamellar structure was retained regardless of the water content. Phosphatidylserine precipitated with

Ca^{2+} had a very dry appearance when viewed under the light microscope and the precipitate could not be completely homogenized. The results are consistent with the picture that Ca^{2+} interacts with the polar group of phosphatidylserine displacing any adsorbed monovalent cations and liberating water from the various hydration shells of the lipid polar group. The final product is an anhydrous (i.e. hydrophobic) metal-lipid complex (cf. ref. 23). The liberation of water on addition of Ca^{2+} indicates that metal and water are essentially competing for the same binding sites. This conclusion is consistent with the hydration studies of Finer and Darke [9] who concluded that the main hydration shell of phospholipids is centered around the phosphate group.

Na^+ also precipitated phosphatidylserine dispersions provided the ion concentration was high enough (see inset of Fig. 1). In contrast to the Ca^{2+} precipitate, the flocculate obtained with Na^+ redissolved readily in excess water. The Na^+ precipitate is not anhydrous as evident from the ^2H spectrum of ^2H_2O in phosphatidylserine/ ^2H_2O/ NaCl mixtures (Table IV). The presence of a doublet indicates that some water is still associated with the lipid structures. An upper limit of approx. 20 mol of water of hydration is obtained from low angle X-ray diffraction studies of a sodium-phosphatidylserine precipitate: a lamellar repeat distance of 60 Å was obtained from this precipitate and this corresponds to the value obtained when about 20 mol water/mol lipid were added to pure phosphatidylserine. We can conclude that the effect of Na^+ on the hydration of phosphatidylserine bilayers is probably intermediate to that of Ca^{2+} and tetraethylammonium ion.

The above interpretation of an anhydrous Ca^{2+} · phosphatidylserine complex is further corroborated by the T_1 measurements in the presence of paramagnetic Mn^{2+} used as an isomorphous replacement for Ca^{2+}. The T_1 of the water protons is reduced by the presence of Mn^{2+}. This is a short range effect which is inversely proportional to the sixth power of the separation of the Mn^{2+} from the water protons. Rapid exchange of water molecules between the hydration sphere of Mn^{2+} and bulk water results in a relaxation rate which is the average weighted over the different environments of the water protons. The observed relaxation enhancement ε^* is given by

$$\varepsilon^* = \frac{Mn_f}{Mn_t}\varepsilon_f + \frac{Mn_b}{Mn_t}\varepsilon_b \tag{3}$$

where Mn_f, Mn_b and Mn_t are the free, bound and total Mn^{2+} concentrations, respectively, and ε_f is the enhancement of free Mn^{2+} which is equal to unity by definition, and ε_b is the enhancement of Mn^{2+} bound to the lipid. The major contribution to the relaxation of the water protons occurs when the water molecule is in the hydration sphere of Mn^{2+}. The enhancement on the relaxation rate $1/T_1$ when paramagnetic ions are bound to macromolecules (first observed with Mn^{2+} bound to DNA by Eisinger et al. [21]) can be ascribed to a reduction in molecular motion of the Mn^{2+} and its hydration shell if the electron spin relaxation time τ_s is long compared with the rotational correlation time τ_r describing the motions of the water protons [21, 24]. The enhancement ($\varepsilon^* > 1$) observed at $[Mn^{2+}] < 10^{-3}$ M (Fig. 3b) is typical for Mn^{2+} being bound to external sites of macromolecules [21, 22, 24]. In this case Mn^{2+} is bound to the phosphatidylserine polar groups at the surfaces of the lipid bilayers. As the Mn^{2+} concentration increased to $[Mn^{2+}] > 10^{-3}$ M the observed ε^* fell rapidly and tended towards 0.8 at $[Mn^{2+}] > 4 \cdot 10^{-3}$ M. This could occur either because all the water had been displaced from the Mn^{2+} · lipid complex or if Mn^{2+}

was bound at a site inaccessible to bulk water. Like Ca^{2+}, Mn^{2+} also precipitated sonicated phosphatidylserine dispersions and its efficacy in raising the turbidity was similar to that of Ca^{2+} shown in the inset of Fig. 1. Comparison of Figs 1 and 3b shows that the decrease in ε^* correlates well with the increase in turbidity or the onset of the precipitation of the Mn^{2+} · phosphatidylserine complex. From low angle X-ray diffraction it is clear that as with other cations the multilamellar structure was also retained in the presence of Mn^{2+}. In conclusion the relaxation enhancement, precipitation and X-ray diffraction experiments with Mn^{2+} are consistent with the formation of an anhydrous Mn^{2+} · phospholipid complex. In turn this leads to the aggregation and fusion of single bilayer vesicles and formation of large multilamellar structures. The properties of the Mn^{2+} · phosphatidylserine complex are very similar to those of the Ca^{2+} · phosphatidylserine complex.

Effects of ions on inter-bilayer interactions

The data in Fig. 1 show that Na^+ and Ca^{2+} decrease the surface charge on phosphatidylserine particles and cause them to flocculate and precipitate. This destabilisation of the colloidal phosphatidylserine dispersion arises because the cations decrease the electrostatic repulsion between particles which arises from overlap of electrical double layers. The total interaction energy arising from van der Waals attraction and electrostatic repulsion between particles can be described in terms of the well known DLVO* theory [25]. Further, the electrolyte concentration at which flocculation should occur can be derived by finding the condition such that the maximum of the potential energy barrier is just zero. The result of this treatment is the so-called Schulze-Hardy rule which states that the flocculation concentration should be inversely proportional to the sixth power of the valency of the flocculating ion [25]. Since the interaction of Ca^{2+} with phosphatidylserine leads to dehydration and formation of a hydrophobic surface, there will be a hydrophobic contribution [19] to flocculation which is not considered in the DLVO theory. As a result, the Schulze-Hardy rule would not be expected to describe exactly the data in Fig. 1 for flocculation of phosphatidylserine particles by Na^+ and Ca^{2+}. In agreement with this, the concentration of Na^+ required to bring the absorbance at 520 nm to 2 is 1.4 M [2] and by the Schulze-Hardy rule the concentration of Ca^{2+} should be $1.4/2^6 = 0.022$ M. In fact, the same absorbance is attained with a Ca^{2+} concentration of 0.009 M [2] which is less than half the concentration predicted. This enhanced effect with Ca^{2+} is a reflection of the displacement of water of hydration by Ca^{2+} and the formation of a hydrophobic surface.

The electrophoretic mobility (ζ-potential) and the surface potential (ΔV) measurements show that both inorganic and organic monovalent cations have similar effects on the surface charge of phosphatidylserine. With the latter type of cations the screening of the surface charge of the lipid is somewhat less effective which might be due to the bulkiness of the tetraalkylammonium ions not allowing close approach and ion pairing of the type observed with inorganic ions. Taking the ζ-potential as a measure of the electrostatic repulsive potential between bilayers then one would expect the tetraalkylammonium ions to precipitate phosphatidylserine dispersions like Na^+ if the decrease in the electrostatic repulsion between phosphatidylserine

* Derjaguin-Landau and Verwey-Overbeek.

bilayers alone were responsible for the precipitation. Clearly another property in addition to the electrostatic screening of the surface charge by counterions is important in determining the resistance of the dispersion to flocculation. An alternative cause of the different behaviour of the two groups of ions could be that the hydration properties of the reaction products (cation-phospholipid) are different. The anhydrous and hydrophobic nature of the Ca^{2+} · phosphatidylserine complex as compared to the tetraethylammonium ion-phosphatidylserine system imposes different macroscopic properties on the lipid bilayer which manifest themselves in the inter-bilayer interactions leading to aggregation and fusion. We therefore have the scheme:

$$\text{single bilayer vesicle} \underset{(C_2H_5)_4N^+}{\overset{Ca^{2+}}{\rightleftharpoons}} \text{multilamellar precipitate}$$

Our results clearly show that with tetraalkylammonium salts the above equilibrium is displaced towards single bilayer structures (vesicles). Since monovalent inorganic and organic cations have more-or-less the same effect on surface charge (Fig. 1), it is not simply electrostatic repulsion between opposing lipid bilayers in the presence of tetraalkylammonium salts which prevents bilayers from approaching closely enough to flocculate and eventually fuse (coalesce) as they do in the presence of Na^+ or Ca^{2+}. Whether this prevention of close-approach (contact) of the bilayers is due to the bulkiness of the tetraalkylammonium ions and/or retention of a hydration shell giving rise to a repulsive potential on close approach is not clear. The repulsion probably contains a contribution from the energy required to displace water molecules from the clathrate layers. It is likely that the different hydration properties of cations, as illustrated in Fig. 2, are important and at least partially responsible for the different bilayer properties in the presence of inorganic and organic cations.

Effects of ions on molecular packing within bilayers

The surface pressure data in Table I show that the packing of phosphatidylserine molecules within a monolayer (or bilayer) is sensitive to the counterion. The general increase in π from the value at zero ionic strength on addition of salt presumably arises from the increased dissociation of the weakly ionizing phosphatidylserine polar group at higher ionic strengths (cf. ref. 26) while the hydrophobic alkylammonium ions give particularly high π because of partial penetration into the monolayer. Increasing the ionic strength further by addition of NaCl can lead to shielding of the charges on the polar groups of the phosphatidylserine and a net reduction in the electrostatic repulsion between adjacent molecules and an increase in the lateral packing density of the bilayer [14]. When Na^+ is displaced from the bilayer by Ca^{2+} a similar condensation occurs [5].

More detailed information about the effect on the packing of Ca^{2+} interacting with the polar group of phosphatidylserine can be obtained from the order parameter of the spin label incorporated into phosphatidylserine bilayers; $S_3 = 0.18 \pm 0.02$ when $[Ca^{2+}] \leqslant 10^{-4}$ M and the change in S_3 paralleled the increase in turbidity (Fig. 1). The correlation time for motion τ_c of the spin label calculated according to the theory of Kivelson [27] showed the same trend with Ca^{2+} concentration as the order parameter. This result shows that the formation of the anhydrous metal-lipid complex affects the packing of the hydrocarbon chains. The interaction in the polar part produces a tighter, more ordered (anisotropic) arrangement of the hydrocarbon chains (cf. ref.

28). At higher Ca^{2+} concentrations line broadening due to spin exchange was observed in the ESR spectrum. This results from clustering of the spin label into islands within the Ca^{2+} · phosphatidylserine matrix [23]. Thus the formation of the Ca^{2+} · phosphatidylserine complex is equivalent to cooling the lipid below the crystal-to-liquid crystal transition temperature so that there is an increase in order ("crystallinity") of the hydrocarbon chains.

Biological implications

We have shown that both intramembrane and intermembrane interactions are sensitive to the nature of the counterion associated with the phospholipid bilayer. On the basis of the differences in hydration and the above effects on colloid stability observed with the two classes of ions, it is to be expected that metal ions such as Ca^{2+} will promote membrane aggregation and fusion (cf. ref. 29) whereas hydrophobic, organic cations will tend to inhibit these phenomena. These differences may have a further biological consequence because whenever a metal ion is displaced by a hydrophobic, organic ion (e.g. a chemical transmitter or drug molecule) the conformation of the membrane should be perturbed. Thus, both the tertiary structure of proteins [30] and the bilayer packing can be altered by such an ion exchange.

ACKNOWLEDGEMENTS

We are grateful to Professors F. Franks and R. J. P. Williams for helpful discussions and suggestions and to Mr. A. Darke for expert technical assistance.

REFERENCES

1 Phillips, M. C. (1972) Prog. Surf. Membrane Sci. 5, 139–221
2 Hauser, H., Phillips, M. C. and Marchbanks, R. M. (1970) Biochem. J. 120, 329–335
3 Rojas, E. and Tobias, J. M. (1965) Biochim. Biophys. Acta 94, 394–404
4 Hauser, H. and Dawson, R. M. C. (1967) Eur. J. Biochem. 1, 61–69
5 Papahadjopoulos, D. (1968) Biochim. Biophys. Acta 163, 240–254
6 Long, C., Shapiro, B. and Staples, D. A. (1962) Biochem. J. 85, 251–256
7 Hauser, H. (1971) Biochem. Biophys. Res. Commun. 45, 1049–1055
8 Finer, E. G., Flook, A. G. and Hauser, H. (1972) Biochim. Biophys. Acta 260, 49–69
9 Finer, E. G. and Darke, A. (1974) Chem. Phys. Lipids 12, 1–16
10 Paltauf, F., Hauser, H. and Phillips, M. C. (1971) Biochim. Biophys. Acta 249, 539–547
11 Finer, E. G. (1973) J. Chem. Soc. Faraday Trans. II 69, 1590–1600
12 Smith, I. C. P. (1972) in Biological Applications of Electron Spin Resonance (Swartz, H. M., Bolton, J. R. and Borg, D. C., eds), pp. 483–539, Wiley, New York
13 Seelig, J. (1970) J. Am. Chem. Soc. 92, 3881–3887
14 Hauser, H. and Phillips, M. C. (1973) J. Biol. Chem. 248, 8585–8591
15 Hertz, H. G. (1963) Ber. Bunsenges. (Phys. Chem.) 67, 311–327
16 Hertz, H. G. and Zeidler, M. D. (1964) Ber. Bunsenges. (Phys. Chem.) 68, 821–837
17 Hertz, H. G. (1973) in Water: A Comprehensive Treatise (Franks, F., ed.), Vol. 3, pp. 301–399, Plenum Press, New York
18 Frank, H. S. and Wen, W.-Y. (1957) Disc. Faraday Soc. 24, 133–140
19 Franks, F. (1975) in Water: A Comprehensive Treatise (Franks, F., ed.), Vol. 4, pp. 1–94, Plenum Press, New York
20 Hauser, H., Phillips, M. C., Levine, B. A. and Williams, R. J. P. (1975) Eur. J. Biochem. 58, 133–144
21 Eisinger, J., Shulman, R. G. and Blumberg, W. E. (1961) Nature 192, 963–964

22 Mildvan, A. S. and Cohn, M. (1963) Biochemistry 2, 910–919
23 Ohnishi, S. and Ito, T. (1974) Biochemistry 13, 881–887
24 Cohn, M. and Leigh, J. S. (1962) Nature 193, 1037–1040
25 Jones, M. N. (1975) in Biological Interfaces, pp. 178–179, Elsevier, Amsterdam
26 Payens, T. A. J. (1957) Proceedings of the 2nd International Congress on Surface Activity, Vol. 1, pp. 64–74, Butterworths, London
27 Kivelson, D. (1960) J. Chem. Phys. 33, 1094–1106
28 Hauser, H. (1975) in Water: A Comprehensive Treatise (Franks, F., ed.), Vol. 4, pp. 209–303, Plenum Press, New York
29 Maeda, T. and Ohnishi, S. (1974) Biochem. Biophys. Res. Commun. 60, 1509–1516
30 Von Hippel, P. H. and Schleich, T. (1969) Acc. Chem. Res. 2, 257–265