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Effect of encapsulated Ca^{2+} on the surface properties of curved phosphatidylcholine bilayers

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In this paper, the influence of Ca^{2+} on the adsorption properties of 1,8-anilinonaphthalenesulfonate (ANS) and analogous probes to sonicated vesicles of phosphatidylcholine was studied by means of spectrofluorometry. The fluorescence of ANS added to the vesicle dispersion increases with the Ca^{2+} concentration in the inner media but remains constant when Ca^{2+} concentration is changed in the outside solution. However, the fluorescence decreases when large anions such as ClO_4^- are present in the external solution. Ca^{2+} inside large liposomes promotes a similar behaviour to that found in sonicated vesicles when they are osmotically contracted in hypertonic media. The results can be interpreted in terms of Ca^{2+} adsorption on the inner interface and a cooperative interaction between the monolayers.

Introduction

The importance of Ca^{2+} in many cellular functions involving membrane systems has stimulated numerous studies to understand the mechanism of binding on phospholipid model bilayers [1–5]. With these purposes the effects of Ca^{2+} have been extensively investigated with several methodologies in membranes composed by acidic or neutral phospholipids such as phosphatidylserine and phosphatidylcholine, respectively. Although Ca^{2+} binding is low on phosphatidylcholine bilayers in comparison to that occurring on negativley charged phospholipids, it presents several interesting features which may be relevant for the understanding of biological functions. Several papers have analyzed the surface potential changes as a function of the lateral pressure in monolayers [6], the surface potential in multilamellar liposomes below and above the gel-liquid crystalline transition temperature, the influence of Na^+ ions [7] and the conformational changes caused by Ca^{2+} binding of the head-group and hydrocarbon chain regions [8,9]. Values for the binding constant and the Ca^{2+}: phospholipid stoichiometry reported in literature are widely different, depending on the region in which the Ca^{2+} action is observed [10]. Binding constants obtained with methods which measure the changes in the conformation of the head groups located in the inner monolayer are different from those measuring the effect on the outer phosphate groups, the choline groups or the hydrocarbon chain region [2,6,8–10]. It is concluded from these evidences that Ca^{2+} binding may affect other groups close to the primary site of interaction. Therefore, it is not unexpected that the Ca^{2+} adsorption is dependent on the type of model membrane system chosen for the analysis.

In sonicated vesicles, both the stoichiometry and the binding constant are different on the inner and the outer monolayers of the bilayer [10,11]. Previous results have shown that Ca^{2+} adsorbs on the inner face causing an increase in the tightening of the bilayer as measured by fluorescence anisotropy. This effect is not observed when Ca^{2+} is added to the external solution [12]. Under this condition, Ca^{2+} adsorption on the outer surface is a function of the Ca^{2+} concentration inside the vesicle [9]. The enhancement of the Ca^{2+} adsorption on the outer interface can be related to a conformational

Abbreviations: PC, phosphatidylcholine; ANS, 1-anilinonaphthalene-8-sulfonic acid; CF, 5(6)-carboxyfluorescein; DPH, 1,6-diphenyl-1,3,5-hexatriene; EDTA, ethylenediaminetetracetic acid; TNS (2,6-TNS), 2-(*p*-toluidinyl)naphthalene-2-sulfonic acid, sodium salt.

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change in the bilayer promoted by the inner Ca^{2+}, involving changes in the surface free energy.

The purpose of this paper is to put into relevance those changes using probes interacting with the monolayer surfaces. For that purpose, a study of the surface properties of Ca^{2+}- and Na^{+}-containing vesicles was performed using ANS and TNS as fluorescent probes.

A comparison of the effect of Ca^{2+} in sonicated vesicle bilayers with those in which curvature is induced by osmotic collapse is made.

Materials and Methods

Egg yolk phosphatidylcholine was obtained from eggs by chloroform/methanol extractions and purified through Silica gel 60 columns. The purity of the collected fractions was checked by thin-layer chromatography (elution solvent chloroform/methanol/water (65 : 25 : 5, v/v)). In all cases a single spot was detected under iodine vapours.

ANS and TNS were obtained from Molecular Probes Inc. All other chemicals were of analytical grade. Bidistilled water was used to prepare all the solutions.

Multilamellar liposomes were prepared by dispersing a dry lipid film in an aqueous solution. Unilamellar vesicles were prepared by sonication of a coarse lipid dispersion in a bath sonicator, (Lab. Supplies Inc. output 100 watts) during 30 minutes. Vesicles external radii were determined by means of dynamic light scattering in an Electronic Coulter Counter. The radius was calculated from the correlation function accumulated after averaging 120 pulses in each sample. At least, three different preparations in each conditions were assayed. The standard deviation of the size was ± 2.5 nm.

The Ca^{2+}- or Na^{+}-containing vesicles were prepared sonicating a lipid dispersion in 0.05 M Ca^{2+} or 0.1 M Na^{+} (chloride salts) buffer 10 mM Tris-HCl (pH 7.4). After a clear solution was obtained, both vesicle samples were dialyzed overnight against 0.1 M NaCl (pH 7.4) under N_2. The absence of Ca^{2+} in the external solution was checked by adding murexide to an aliquot of the dialyzed sample [13]. When the spectra of murexide in the dialyzed sample was identical to that found in a NaCl solution with EDTA the dialysis was considered complete.

Aliquots of the two vesicle types were diluted in 0.1 M NaCl solution and titrated with a 10^{-2} M ANS solution.

Fluorometric assays were done in a Perkin-Elmer 2000 spectrofluorometer at 470 nm and 372 nm for the emission and excitation wavelength, respectively.

TNS has a significant absorbance at both the excitation and emission wavelengths. These absorbances attenuate the excitation and emission by $10^{-A_{ex}/2}$ and $10^{-A_{em}/2}$. This is called an inner filter effect. The corrected fluorescence intensity is approximately given [14] by:

$$F_{corr} = F_{obs} \text{ antilog}(A_{ex} + A_{em})/2$$

Fluorescence anisotropy was determined using diphenylhexatriene (DPH) as a membrane probe. Samples were incubated in darkness after the addition of the probe in tetrahydrofurane during 1 h. After the measure of $I_{\perp}$ and $I_{\parallel}$ the anisotropy values (Γ) were calculated according to the relation:

$$\Gamma = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

For details of the Γ parameter determinations in Ca^{2+} vesicles see Ref. 12.

The trapping of carboxyfluorescein (CF) in vesicles containing 0.05 M Ca^{2+} or 0.1 M Na^{+} was obtained by dispersing the lipids in a 50 mM CF solution [15]. At this concentration CF is self quenched. Control experiments showed that CF fluorescence is unchanged by the Ca^{2+} concentrations used in this work. This is in agreement with previous reports [19]. The external CF after the vesicles were prepared was eliminated by elution through a Sephadex G-75 column. The trapped fluorophore was determined by measuring the fluorescence at 520 nm (emission wavelength) before and after the addition of 100 μl of a 10% (w/w) Triton X-100 solution.

The phospholipid concentration of the sample was determined by phosphate analysis.

Results

When ANS and TNS is added to a vesicle suspension a different stationary value of fluorescence is obtained after 40 min of incubation with both dyes depending on the vesicles contain either Ca^{2+} or Na^{+} solutions (Fig. 1). For this reason, all the experiments were done after incubation of the vesicles with the fluorophore one hour before measurement.

The double-reciprocal plot of the titration curves of Ca^{2+}- and Na^{+}-vesicles with ANS indicate that the fluorescence obtained with Ca^{2+}- and Na^{+}-vesicles converges to the same value at high ANS concentrations. In addition it is observed from the intersection on the abscissa that the ANS affinity of Na^{+}-vesicles is lower than those containing Ca^{2+} (Fig. 2).

The ANS fluorescence obtained with Ca^{2+} and Na^{+} vesicles is independent on the external Ca^{2+} concentration (Fig. 3). However, the fluorescence of Ca^{2+}- and Na^{+}-vesicles depends on the type of anion in the external solution (Fig. 4). The variation of the ANS fluorescence intensity with ClO_4^- ion concentration, maintaining the same osmotic balance, is illustrated in Fig. 4 by

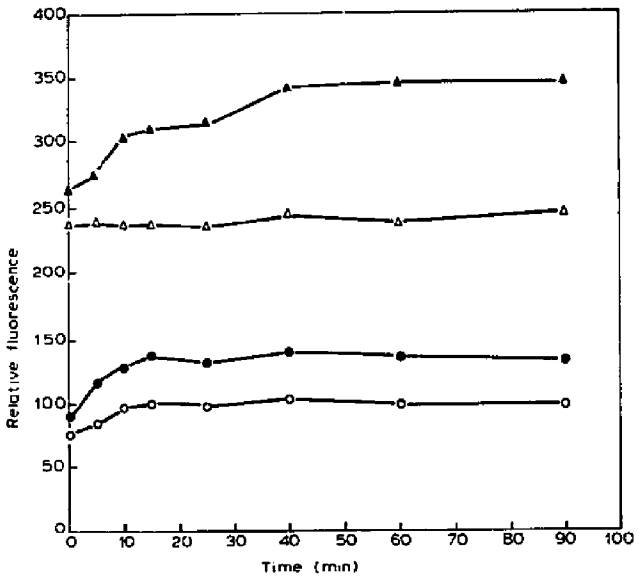

Fig. 1. Increase of fluorescence in the presence of Na^+- and Ca^{2+}-containing vesicles. Addition of $2.5 \cdot 10^{-5}$ M ANS to a suspension of vesicles containing 0.1 M Na^+ (○) or 0.05 M Ca^{2+} (●). Total lipid concentration was 0.52 mM. (△,▲) correspond to similar experiments using TNS. EDTA was added to the outer solution to sequest external Ca^{2+}. Control experiments showed no effect of EDTA on fluorescence measurements.

the straight-line plots of $F_0/(F_0 - F)$ vs. $[ClO_4^-]$ for Na^+- and Ca^{2+}-vesicles. It is observed that the slopes, which are proportional to the dissociation constants of ClO_4^- widely differ from one type of vesicle to another.

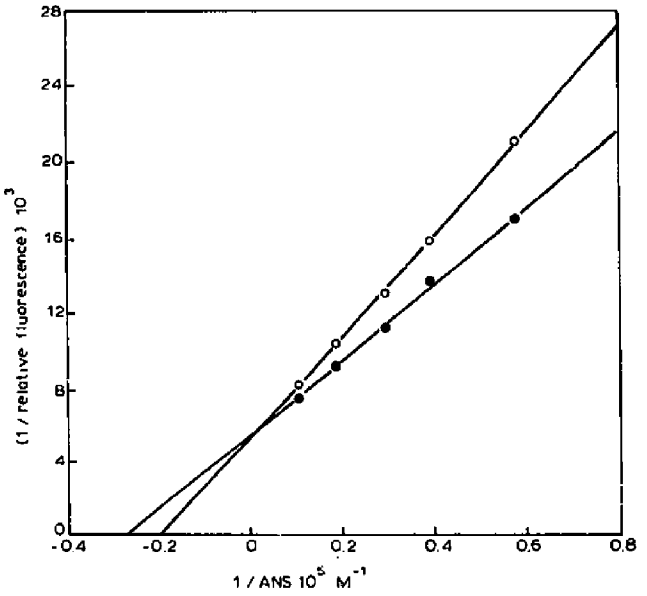

Fig. 2. Double-reciprocal plot of the titrations of Ca^{2+}- and Na^+-vesicles with ANS. Ca^{2+}- (●) and Na^+- (○) vesicles containing a 0.1 N solution inside were dialyzed against 0.1 M NaCl previous to ANS addition. Vesicles were incubated 1 h in the presence of the dye before the fluorescence value was taken. Titrations were done by duplicate with a total concentration of lipid of 0.52 mM at 25 °C.

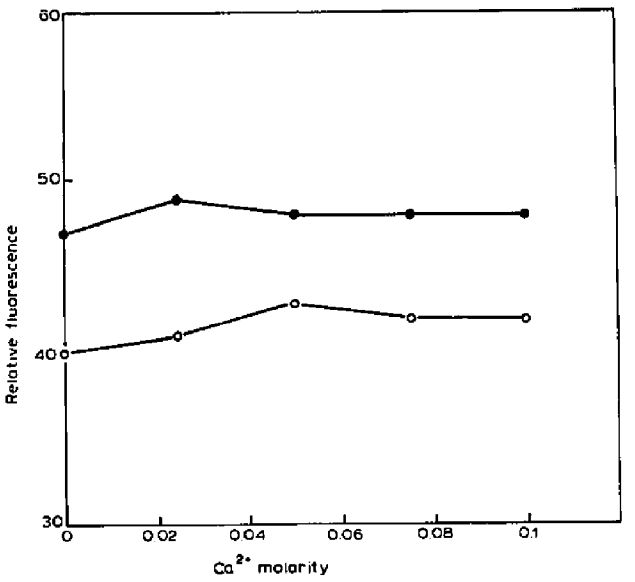

Fig. 3. Effect of the external Ca^{2+} medium on ANS fluorescence of Ca^{2+}- and Na^+-containing vesicles. ●, Ca^{2+}-vesicles dispersed in $CaCl_2$; ○, Na^+-vesicles dispersed in $CaCl_2$.

In both cases the fluorescence values attained at the ordinate axis indicate that a fraction of the ANS fluorescence still remains at high ClO_4^- concentrations. In this condition the fluorescence difference found between Na^+- and Ca^{2+}-vesicles depends on the size of the anion present in the external solution (Fig. 5).

Differences in fluorescence with Ca^{2+}- and Na^+-vesicles are also observed when TNS, a probe that does not penetrate the phosphatidylcholine vesicles within

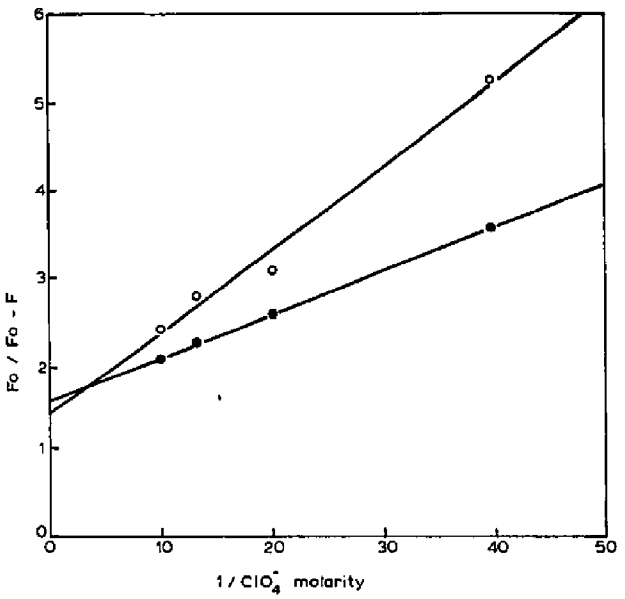

Fig. 4. Double-reciprocal plot of the decrease of relative ANS fluorescence by perchlorate according to the equation $(F_0 - F)/F_0 = f_0(Q)/K(Q) + [Q]$ (see Discussion, p. 119). Ca^{2+}- (●) or Na^+- (○) containing vesicles were dispersed in $NaClO_4$ solutions.

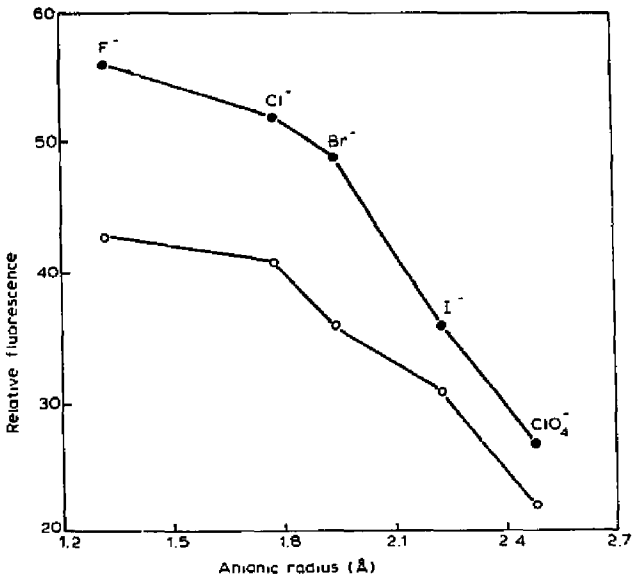

Fig. 5. Relative fluorescence of Ca^{2+}- (●) and Na^{+}- (○) containing vesicles as a function of the radius of the anionic species present in the external media. All assays were done using a 0.1 N concentration of the salts and at 25°C.

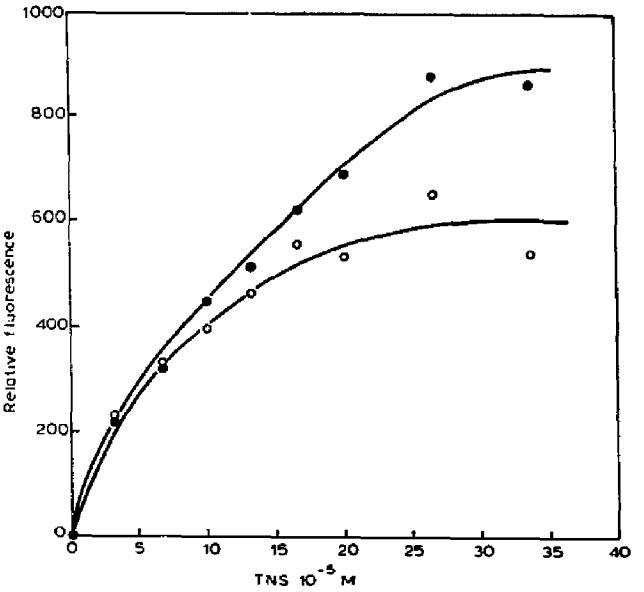

Fig. 6. Titration of Na^{+}- and Ca^{2+}-vesicles with TNS. Similar vesicles than Fig. 2 were titrated with TNS solution. ●, Ca^{2+}-vesicles; ○, Na^{+}-vesicles. The values were corrected for the inner filter effect (see Materials and Methods).

less than 1 h [16], is used (Fig. 6). Considering the results of Fig. 1 in which an stationary value of fluorescence is found for both types of vesicle after 45 min, the measurements were made after one hour of incubation. Taking into account the data in Fig. 3, the differences in Fig. 6 would depend on the Ca^{2+} concentration inside the vesicle and not on the external Ca^{2+} concentration.

When a solution containing $2.5 \cdot 10^{-5}$ M ANS is titrated with Na^{+}- or Ca^{2+}-vesicles the double-reciprocal plots indicate that the same number of phospholipids combines with ANS in both types of vesicles (Fig. 7). The ANS binding constants are larger for Ca^{2+} than for Na^{+} vesicles and the number of sites are equal in both cases (Table I). Thus, the changes observed with ANS and TNS should be accomplished with physical changes in the bilayer vesicle that would influence the dye affinities of the sites.

The trapping and size data shown in Table I indicate that Na^{+}-vesicles are smaller than those containing Ca^{2+}. The enlargement of the vesicle by Ca^{2+} makes

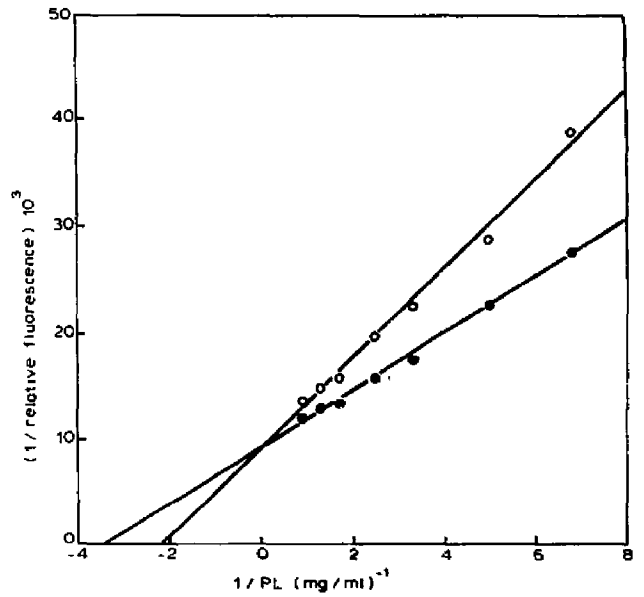

Fig. 7. Double-reciprocal plots of the titration of ANS solutions with Na^{+} (○) and Ca^{2+} (●) vesicles in the presence of chloride.

TABLE I

Physical parameters of Na^{+}- and Ca^{2+}-containing vesicles and the ANS fluorescence corresponding to the inner and the outer interface

Standard deviation (S.D.) of r: ±0.002. CF/PL trapping of CF. ϕ, vesicle diameter as determined by dynamic light scattering (S.D. = ±2.5 nm). δ, thickness of the bilayer calculated from the external and internal radius. r, fluorescence anisotropy. F_{int}, fluorescence obtained in presence of 0.1 M Na ClO_4 in the external media. F_{ext}, fluorescence obtained from the difference between total fluorescence in presence of 0.1 M NaCl and F_{int}. $K_{dis}(Cl^{-})$ and $K_{dis}(ClO_4^{-})$, dissociation constants of ANS in the presence of Cl^{-} and ClO_4^{-}, respectively.

	CF/PL (mol/mol)	ϕ (nm)	δ (nm)	r	F_{int}	F_{ext}	$K_{dis}(Cl^{-})$ (M)×10⁵	$K_{dis}(ClO_4^{-})$ (M)×10⁵
Ca^{2+}	21.7	40	6.0	0.112	27	25	4.9	6.2
Na^{+}	13.6	38	8.0	0.90	22	19	7.31	5.9

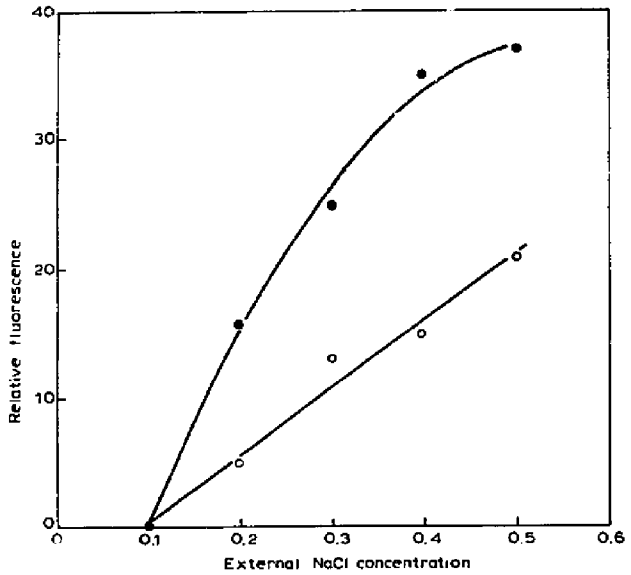

Fig. 8. Effect of shrinkage on the ANS binding of Na^+- and Ca^{2+}-containing liposomes. Liposomes were prepared in 0.05 M $CaCl_2$ solutions (●) or 0.1 M NaCl solutions (○) and dispersed in solution of NaCl concentrations indicated in the abscissa.

the bilayer to become thinner with an increase in the rigidity as measured by DPH. It must be noticed that within the experimental error the change in the external size by Ca^{2+} inside is only 5%. However, the change in thickness is 25% a value near to the change observed in the trapped volume. This indicates that the thickness may vary by the interaction of the Ca^{2+} ion with the inner surface, promoting the increase in the bilayer rigidity. An effect of the Ca^{2+} concentration on the

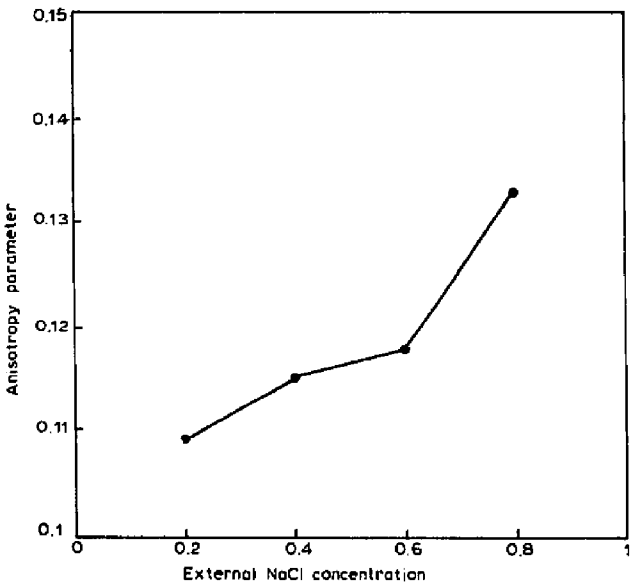

Fig. 9. Effect of shrinkage on the fluorescence anisotropy measured with DPH for Ca^{2+}-containing liposomes. The external solutions were similar to those used in Fig. 8.

fluorescence efficiency can be discarded as mentioned in Materials and Methods.

The comparison of the K_{dis} values of ANS obtained in the presence of ClO_4^- for Na^+- and Ca^{2+}-vesicles in Table I indicates that the ANS affinity is not affected by the internal Ca^{2+} when ClO_4^- is added to the external solution. Thus, the same independence of Ca^{2+} as found in Fig. 3 for the external Ca^{2+} concentration is obtained with internal Ca^{2+}. The increase of rigidity promoted by the internal Ca^{2+} seems to affect the ANS affinity on the external surface but not that corresponding to the internal monolayer. This can be inferred from the difference in the K_{dis} values obtained in the presence of Cl^-. In this case, the affinity would correspond to a mean value of the internal and the external surface. The difference can be ascribed to the external surface because in the presence of ClO_4^- no significant differences are found. In Table I it can also be observed that the fluorescence corresponding to the internal (F_{int}) and the external (F_{ext}) surfaces are similar in Ca^{2+}- and Na^+-vesicles. In Na^+-vesicles this can be ascribed to the fact that internal and external surfaces are in contact with Na^+. However, in the case of Ca^{2+}-vesicles the internal is in contact with Ca^{2+} and the external with Na^+. If the fluorescence would be a consequence of the ionic composition of the solution in contact with the membrane surface, the F_{ext} in Ca^{2+}-vesicles should be equal to that found with F_{int} and F_{ext} in Na^+-vesicles.

Interestingly, a similar physical change is observed when large liposomes are subjected to an osmotic volume contraction (Fig. 8). In these cases, multilamellar liposomes containing Na^+ or Ca^{2+} were subjected to hypertonic shrinkage by dispersing them in NaCl solutions of increasing tonicity. It is observed that the ANS fluorescence of Ca^{2+}-liposomes increases more rapidly than that of those containing Na^+ for the same osmotic gradients. In Fig. 9, it is observed that the increase in ANS fluorescence on Ca^{2+}-containing liposomes is accompanied by an increase in anisotropy measured with DPH induced by the osmotic shrinkage.

Discussion

The double-reciprocal plots of the fluorescence data obtained with Ca^{2+}- and Na^+-vesicles indicate that the number of ANS sites and the number of phospholipid molecules involved in the ANS–membrane interaction are comparable in both types of vesicle. The lipid/ANS ratio can be calculated as follows. The lipid concentration corresponding to a fluorescence value for which all the accessible lipid surfaces are saturated with ANS is divided by the ANS concentration corresponding to the same value of fluorescence in which it can be assured that all the ANS is bound to the vesicle due to an excess of vesicles. Thus, the lipid/ANS ratio for both types of vesicle is 18.

However, the ANS binding constant for Ca^{2+}-vesicles is higher than for Na^+-vesicles and decreases when large anions such as ClO_4^- are present in the external medium. Notwithstanding, at high anion concentration, fluorescence is not completely eliminated. This can be interpreted as a consequence that ClO_4^- can only compete with ANS on the external vesicle interfaces because it does not permeate the vesicle bilayer. The remaining fluorescence would correspond to ANS adsorbed on the internal surface. A certain fraction of fluorescence is associated with the ANS on the inner surface which is inaccessible to ClO_4^- (f_i). The intercept $1/f_0$ in Fig. 4 is, according to the equation

$$\frac{F_0}{F_0 - F} = \frac{K(a)}{f_0(a)} = \frac{1}{f_0}$$

the inverse of the fractional accessible sites for ClO_4^-, where F_0 is the fluorescence in the absence of ClO_4^-, F the fluorescence at each ClO_4^- concentration [Q], and K(Q) the dissociation constant of ClO_4^-. The value for f_0 obtained from Fig. 4 is 0.66 denoting that 66% of the fluorescence corresponds to the external interface. This percentage is similar to that of lipids in the outer leaflet of the vesicle bilayer.

In Table I the fluorescence corresponding to the external and the internal vesicle surface are calculated. In the presence of Cl^- or F^- competition with ANS on the external surface is negligible. Thus these values can be taken as the fluorescence of the internal (F_i) plus the external (F_e) surfaces. At high ClO_4^- concentration, considering that these anions cannot penetrate into the vesicles, the fluorescence values correspond to the internal interface. The difference between F_t and F_i can be ascribed to the fluorescence corresponding to the external surface. According to the values in Table I, ANS binding on the external surfaces of Ca^{2+} vesicles are higher than those in the external surfaces of Na^+ vesicles. This is independent on the ionic composition of the external solution (Fig. 3). Moreover, the observations with TNS, which hardly permeates the bilayer [16], permits to derive the same conclusion. The presence of an asymmetric membrane concentration of ClO_4^- may generate a large diffusion potential due to osmotic water extrusion if this anion is not permeant. The effect of osmosis on vesicles and liposomes in relation to the effects on the membrane structure in the presence of Ca^{2+} is analyzed in Figs. 8 and 9 and Table II.

It is interesting to observe that in Na^+-vesicles the fluorescence values corresponding to the internal and the external surfaces are comparable between them and both are lower than those obtained with Ca^{2+}-vesicles. This can be ascribed to the presence of Na^+ at both interfaces. In the case of Ca^{2+}-vesicles inner fluorescence values are higher than that for the same interface with Na^+. This is not unexpected since ANS would

TABLE II

Effect of osmotic stress on the anisotropy parameter of vesicles and liposomes containing 0.05 M Ca^{2+} solutions

Hypertonic stress was achieved dispersing the vesicles or liposomes in 1 M NaCl. Standard deviation of $r = \pm 0.002$.

	Iso-osmotic condition	Under hypertonic stress
Vesicles	0.112	0.128
Liposomes	0.110	0.133

adsorb more easily on surfaces in which positive charges have been promoted by adsorbed Ca^{2+}. In these vesicles, the outer surface is in contact with a Na^+ solution after extensive dialysis (see Materials and Methods). Thus, a similar value to that obtained with Na^+ vesicles would be expected. However, it is seen that the values for the external fluorescence are comparable to those with the internal. There are two possible explanations for this phenomenon. Ca^{2+} is exchanged by Na^+ in the dialysis or the internal Ca^{2+} is promoting by its interactions on the internal surface a change in the properties of external surface of the vesicles. Moreover, if Ca^{2+} is leaked during dialysis it would be expected that it would also be absent in the external solution as confirmed by the Murexide assay. If this would be the case the structural changes would still remain in the light of rigidity, size and fluorescence assays. Considering that leakage is negligible under these circumstances [17] and that inner Ca^{2+} promotes physical changes in the bilayer structure, such as an increase in rigidity, it is possible to argue that the presence of internal Ca^{2+} may affect the surface properties. The rigidization and reduction in thinness of the bilayer might cause a slight displacement of the choline groups $(N^+(CH_3))_3$ plane with respect to that of the PO_4^{3-} groups [2,3]. Under these conditions, the positive charges would be in an external plane with respect to the imaginary plane passing through the center of the bilayer. Thus, the binding of negative charges will be enhanced because the positive groups are less unshielded. This picture also explains the aggregation of Ca^{2+} vesicles caused by ferricyanide in the presence of external Ca^{2+} [18].

The simplest way to describe Ca^{2+} adsorption is by the isotherm

$$\frac{(1-\theta)}{\theta} = \frac{K}{[Ca]^n} \qquad (1)$$

When $n = 1$ the isotherm fulfills that of Langmuir. In the case $n \neq 1$, the adsorption takes place with changes on the adsorptive surface. The propagation of these changes in the surface into the inner regions of the bilayer depends on the strength of the Ca–phospholipid interaction. In our case, this propagation is visualized by an increase in the bilayer anisotropy. This phenome-

non appears when the bilayer becomes curved either by sonication or by osmotic compression. As pointed out previously Ca^{2+} adsorbing on the inner monolayer is stronger than that on the outer, the stoichiometry of the former being 2:1 lipid/Ca^{2+}. According to Eqn. 1 this stoichiometry does not fulfill a Langmuir isotherm. The effect of adsorption on the structure can be described by a conformational change, the free energy of which ($\Delta G'$) is related to n by

$$-\frac{1}{RT}\frac{\partial \Delta G'}{\partial \ln C} = (n-1) \quad (2)$$

Thus, $n = 0.5$ implies an increase in the excess of free energy of the system when Ca^{2+} concentration increases on the inner surface.

This excess of free energy would be the factor by which adsorption phenomena on the outer surface would be enhanced when Ca^{2+} is inside the vesicle [9].

The progress of the perturbation from the inner to the outer monolayer might be related with the distribution of kinks in the hydrocarbon chain region as suggested by fluorescence anisotropy determinations (Table I). A detailed study of the effect of internal Ca^{2+} on the bilayer rigidity has been published elsewhere [12].

It is interesting to note that the values of the rigidity observed when the liposomes filled with Ca^{2+} are collapsed in hypertonic media are very similar to those obtained when sonicated vesicles containing Ca^{2+} are subjected to the same osmotic stress (Table II). In the first case, Ca^{2+} concentration increases in the inner solution and the curvature of the bilayer are obtained in one step. In the second, curvature is achieved by sonication and Ca^{2+} is increased by the osmotic shock.

A similar effect on membrane properties is observed independently the way followed to obtain an asymmetric membrane packing and distribution of adsorbed species. As the increase in anisotropy accompanies the increase of ANS adsorption in the presence of inner Ca^{2+} in sonicated vesicles and in liposomes collapsed by osmotic shrinkage the coupling of curvature and the effect of Ca^{2+} adsorption in the inner interface seems to be a more general phenomenon than a peculiar property of sonicated vesicles.

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