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Leakage from egg phosphatidylcholine vesicles induced by Ca^{2+} and alcohols

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The results shown in this paper indicate that the permeability properties of egg yolk phosphatidylcholine sonicated vesicles as detected by the leakage of carboxy fluorescein changes according to the Ca^{2+} content. Vesicles containing Ca^{2+} show a higher rate of leakage than those containing Na^+ solutions in response to the increase of Ca^{2+} concentration in the outer solution. The results are interpreted in terms of the rigidity promoted by Ca^{2+} and are compared to those obtained with long and short chain alcohols.

Introduction

The decrease of the stability of experimental model systems of lipid membranes results in a lower trapping efficiency, non specific liberation, bursting, mechanical fragility or enzymatic susceptibility. Lipid composition, type of aqueous solutes in the dispersing solution, defects in the packing of the phospholipids, adsorptive and permeability properties of the bilayer are some of the factors usually involved [1–12].

The problem of stability in lipid model membranes has been related to the presence of charges and the degree of saturation of the constituent phospholipids. Both factors are affected by the presence of Ca^{2+} which, as is known, binds to acidic phospholipids and in certain degree to neutral phosphatidylcholines (PC) in the gel state [13,14]. Planar membranes of PC do not show any sign of instability with an asymmetric distribution of Ca^{2+} up to 200 mM on one side and none on the other. In contrast, the stability of membranes containing net acidic phospholipids such as phosphatidylserine (PS) depends on the extent of the chelation of the head groups by Ca^{2+} at each side of the membrane [15,16].

In the case of small sonicated vesicles it could be possible that the stability is a consequence of the different packing of the phospholipids at both sides of the bilayer as a result of the bilayer curvature.

Saturated phosphatidylcholine sonicated vesicles present higher leakage and fusion than those prepared with unsaturated lipids such as egg PC. This difference may be ascribed to the number of defects appearing in the bilayer as a consequence of the sonication. In general, vesicles of dipalmitoylphosphatidylcholine (DPPC) prepared below the gel-liquid crystalline transition temperature are more unstable than those prepared with the membrane in the liquid state [17,18]. In addition, interfacial properties of vesicle bilayers differ from those of liposomes as demonstrated by the polar solutes and Ca^{2+} adsorption [19,20].

The purpose of this paper is to report experimental results showing that sonicated vesicles of egg PC are unstable and leaky when they are

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prepared above the transition temperature in contact with Ca^{2+} solutions. The magnitude of the leakage is a function of the Ca^{2+} distribution between the inner and the outer solution the Ca^{2+} concentration and the input of stirring.

Similarly, the leakage of the fluorophore induced by long and short chain alcohols is also a function of the Ca^{2+} concentration present at each side of the bilayer. These results are indicative that the stability of a lipid membrane constituted of single neutral phospholipids also depends on the concentration of compounds inside the vesicle and on the type of solutes in the external solution.

Materials and Methods

Egg yolk phosphatidylcholine was obtained from eggs according to standard procedures and purified through a silica gel column. The purity of the samples was checked by thin-layer chromatography–only one spot was detected when they were run with a chloroform/methanol/water mixtures (65 : 35 : 8, v/v). Carboxyfluorescein was from Eastman-Kodack and purified through LH-20 Sephadex column following the procedure described by Ralston et al. [21]. All other chemicals were of analytical degree and the water was twice distilled.

Liposomes were prepared by dispersing in aqueous solutions a dry film of egg PC obtained after evaporating the solvent under N_2 stream. All the solution contained 10 mM Tris-HCl or Hepes buffer (pH 7), 50 mM 6-carboxyfluorescein and the desired Ca^{2+} or Na^+ concentrations. At this concentration carboxyfluorescein is self-quenched and the release of the quenching is obtained by diluting the fluorophore below 10 mM [22].

The course lipid dispersion was sonicated in a bath sonicator (Laboratory Supplies Inc.) at 100 watts during 30 min at room temperature.

Previous to the sonication samples were bubbled with N_2 and sealed. Care was taken in order to maintain the temperature of the bath below 20 °C.

After the sonication, vesicles were annealed at room temperature for at least half an hour. Then, the external carboxyfluorescein was removed by eluting 100 μl through a Sephadex G-50 column with isotonic NaCl-buffer solution.

A brownish fraction of around 0.5 ml was collected at the front of the elution. Free carboxyfluorescein ran behind.

Control experiments in order to check the homogeneity of the sample were performed running unsonicated liposomes and sonicated vesicles loaded with carboxyfluorescein in a Sepharose 2B column. It was observed that after the sonication procedure described above a negligible fraction of coarse liposomes was present.

The leakage experiments were performed in a SLM 4100 spectrofluorometer (excitation wavelength 490 nm; emission wavelength 520 nm) with thermostatized cuvette holder and magnetic stirring. An aliquot of 20 μl of the carboxyfluorescein containing vesicles was diluted in 2 ml of the corresponding test solution.

A serie of control experiments showed that the reproducibility of the results greatly depended on the weight and shape of the magnetic bar chosen. Therefore, the effect of stirring was tested as follows. The vesicles were dispersed in the cuvette solution and hand-shaked (two or three ups and downs) and immediately introduced in the cuvette holder. Fluorescence was followed during a period of time (see Fig. 1) after which the magnetic bar was introduced. In all experiments similar shaped and weighted bars were used at a fixed stirring speed. No quantification of the r.p.m. was intended. All experiments were done at 25 ± 0.1 °C.

The data was collected in an attached Apple II computer and stored for future analysis and calculation.

The percentage of leakage was calculated by means of the relation

$$\% \text{ leakage} = \frac{A}{A_\infty} \times 100 \qquad (1)$$

where A_∞ and A correspond to the fluorescence values obtained by adding Triton and that found in the outer media at different times, respectively.

The total amount of trapped carboxyfluorescein was calculated from the fluorescence obtained after the addition of 10 μl of a 10% (w/w) Triton X-100 solution.

The kinetic release of the fluorophore can be expressed as a first-order process by

$$-\partial A_i/\partial t = k_{rel} A_i \qquad (2)$$

where A_i is a function of the inner fluorophore concentration. The integration of Eqn. 2 considering that $A_i = A_\infty - A$ results in

$$\ln \frac{A_\infty - A}{A_\infty} = - k_{rel} t \quad (3)$$

where k_{rel} is the release rate constant.

A typical plot of Eqn. 3 is shown in Fig. 3. Permeabilities were calculated by the equation [23]

$$P = k_{rel} \frac{v_i}{a_i} \quad (4)$$

where v_i and a_i are the internal volume and area, respectively.

v_i and a_i were calculated assuming a spherical shape vesicle of radius r_i. This was deduced from the external diameter values obtained by dynamic light scattering determinations. The diameter values corresponding to Ca^{2+} vesicles was 380 Å and to Na^+ vesicles 200 Å. The membrane thickness was taken as 61 Å according to the differences between trapped volumes and that deduced from size measurements.

Details of these calculations and the size dependence with the Ca^{2+} concentration will be published elsewhere.

Results

The onset of stirring of a dispersion of sonicated vesicles trapping 0.050 M Ca^{2+} solution and 6-carboxyfluorescein promotes a release of the fluorophore (Fig. 1).

In the same conditions of stirring as above the amount of leakage markedly depends on the ionic composition of the solutions inside and outside the vesicles.

In Fig. 2, we compare the leakage of vesicles prepared in 0.050 M $CaCl_2$ (A) and 0.10 M NaCl (B) solutions dispersed in different concentrations of $CaCl_2$ and NaCl. It can be observed that upon stirring, Ca^{2+}-containing vesicles are more leaky than those containing Na^+ when Ca^{2+} is present in the outer solution.

The vesicles seem to respond to outer Ca^{2+} according to the inner Ca^{2+} concentration.

The effect of external Ca^{2+} on vesicles containing 0.025 M $CaCl_2$ inside is observed in Fig. 3.

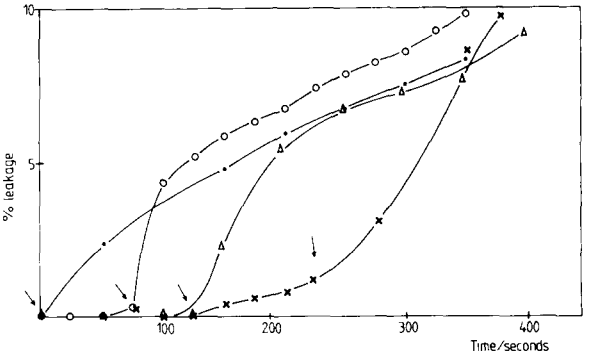

Fig. 1. Leakage of egg PC sonicated vesicles containing 50 mM Ca as a function of time. Arrows indicate the time at which stirring was accomplished by introducing a magnetic bar in the cuvette. Percentage of leakage and experimental conditions are described in Material and Methods.

The rate of leakage is higher when outer Ca^{2+} concentration is increased. Under the same conditions leakage from Na^+-containing vesicles is negligible and it is not enhanced by increasing the Ca^{2+} concentration in the outer solution (Fig. 2B).

It must be noticed that hypertonic concentrations of 0.5 M Na^+ promote a lower leakage than that found in 0.5 M $CaCl_2$ (see Fig. 2A). In addition, data of Table I show the same permeability values for Na^+ vesicles dispersed in 0.1 M, 0.5 M NaCl or 0.5 M $CaCl_2$. Thus, although

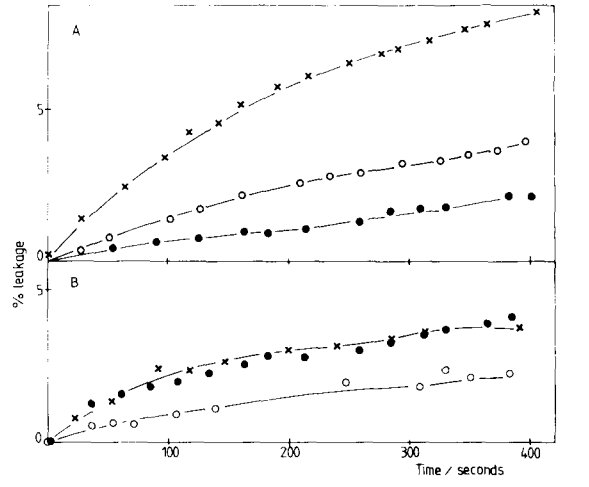

Fig. 2. Leakage of (A) Ca^{2+} containing vesicles and (B) Na^+ containing vesicles dispersed in 0.1 M NaCl (●); 0.5 M NaCl (○) and 0.5 M $CaCl_2$ (×). Stirring was accomplished with a magnetic bar with the precautions described in the experimental section.

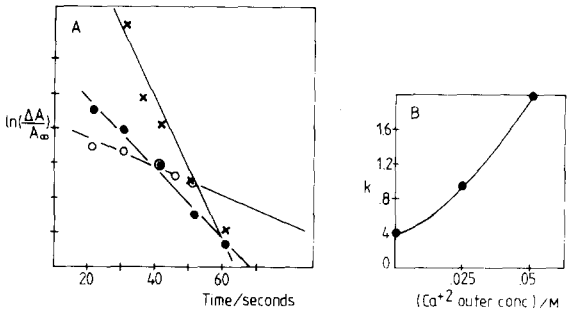

Fig. 3. Kinetic of leakage of egg PC vesicles containing 0.025 M $CaCl_2$ in 0.5 M $CaCl_2$ (×); 0.025 M $CaCl_2$ (●) and 0.1 M NaCl (○). (A) Semilogarithmic plot of the leakage as a function of time. (B) Specific rate constant of leakage at different Ca^{2+} concentration in the outer solution.

TABLE I

KINETIC PARAMETER AND PERMEABILITY VALUES FOR VESICLES WITH DIFFERENT CONTENTS OBTAINED IN THE PRESENCE OF STIRRING

For calculations see Materials and Methods.

Inner media	Outer media	k release (s^{-1})	P (10^{-11} cm/s)
0.1 M Na^+	0.5 M Ca^{2+} + hexadecanol	2.25	96
0.05 M Ca^{2+}	0.1 M Na^+ + hexadecanol	0.75	34.75
0.05 M Ca^{2+}	0.5 M Ca^{2+}	0.36	16.68
0.05 M Ca^{2+}	0.5 M Ca^{2+} + hexadecanol	0.36	16.68
0.1 M Na^+	0.5 M Ca^{2+}	0.27	11.61
0.1 M Na^+	0.5 M Na^+	0.27	11.61
0.1 M Na^+	0.1 M Na^+	0.27	11.61

osmotic concentration could contribute to a slight increase of leakage (compare data for 0.1 M NaCl with that for 0.5 M NaCl in Fig. 2A) the release seem to be caused by the presence of Ca^{2+}.

In particular, the specific rate constant of the leakage process for Ca^{2+} vesicles for different Ca^{2+} concentrations in the outer media are compared in Fig. 3B.

The influence of Ca^{2+} on the permeability of egg PC vesicles, is also found when compounds affecting the hydrocarbon core of the bilayer are present in the solution.

In this direction hexadecanol and butanol were tested in the presence and in the absence of Ca^{2+} at both sides of the membrane. The first one incorporates into the bilayer increasing its rigidity, the second, permeates the bilayer increasing its fluidity [24]. It can be observed in Fig. 4A that the presence of hexadecanol does not promote leakage from Na^+-containing vesicles dispersed in Na^+ solutions. However, the leakage is enhanced by hexadecanol when Na^+ vesicles are dispersed in 0.5 M Ca^{2+} solution. In this case, outer Ca^{2+} induces leakage from Na^+ vesicles in a higher magnitude to that found for Ca^{2+}-containing vesicles in the absence of the alcohol (see Fig. 4B and Table I).

This suggests that leakage is linked, at least in part, to the interaction of Ca^{2+} with the outer surface of the vesicle. This interaction may be related to the adsorption of Ca^{2+} which, as is known, is enhanced in rigid bilayers. The rigidity, in the cases analyzed here, may be promoted either by the inner Ca^{2+} (Fig. 2) or by the incorporation of hexadecanol (Fig. 4A). The effect on leakage of the simultaneous presence of these

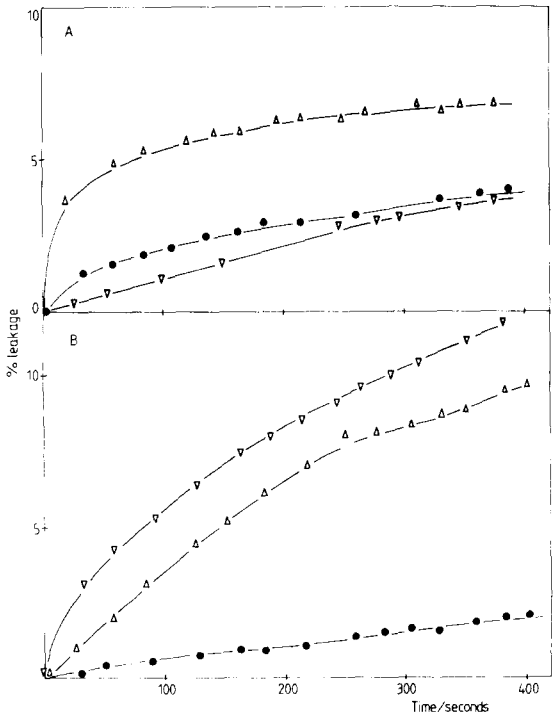

Fig.4. Influence of hexadecanol on the leakage from egg PC vesicles containing 0.1 M NaCl (A) and 50 mM $CaCl_2$ (B) in different external solutions. ●, 0.1 M NaCl; ▽, hexadecanol in 0.1 M NaCl; △, hexadecanol in $CaCl_2$ 0.5 M. The hexadecanol concentration was 0.017 M.

two membrane rigidity inducing factors was assayed next. Fig. 4B shows that the long chain alcohol does not promote significant increase in the leakage when added to Ca^{2+} vesicles in the presence of Ca^{2+} in the outer solution.

Except for a higher slope, Ca^{2+} vesicles dispersed in 0.5 M Ca^{2+} present the same magnitude of leakage in the absence or in the presence of hexadecanol. However, in the same figure it is observed that hexadecanol promotes leakage from Ca^{2+} containing vesicles in the absence of Ca^{2+} in the external solution. Here, it is again observed that the effect of the membrane rigidity inducing factor, in this case hexadecanol, does not depend on the osmotic concentration of the inside and the outside media.

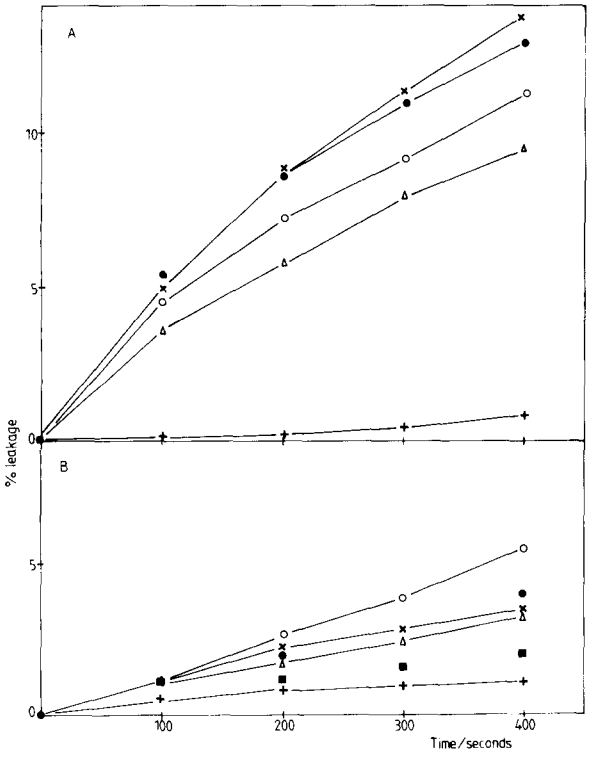

Fig. 5. Influence of 0.1 M butanol on the leakage from egg PC vesicles containing 0.1 M NaCl (A) and 50 mM $CaCl_2$ (B) in different Ca^{2+} concentrations. These experiments were performed at 30 °C and without stirring. +, Control without butanol and external Ca^{2+}; ●, with 0.1 M butanol in the absence of Ca^{2+} in the external solution; ×, with 0.1 M butanol plus 1 mM Ca^{2+} in the external solution; ○, with 0.1 M butanol plus 20 mM Ca^{2+} in the external solution; △, with 0.1 M butanol plus 40 mM Ca^{2+} in the external solution; ■, with 0.1 M butanol plus 80 mM Ca^{2-} in the external solution.

The magnitude of the leakage induced by Ca^{2+} in the absence of stirring is very low as can be observed in Fig. 1. However, as shown in Fig. 5 (A and B) Ca^{2+} affects the leakage induced by 0.1 M butanol in the absence of permanent stirring.

The effect of butanol is much more significant on Na^+ than on Ca^{2+} vesicles. That is, butanol seems to induce more leakage in vesicles whose inner bilayer interface is in contact with Na^+. This leakage is even higher than that found for Ca^{2+}-containing vesicles in Ca^{2+} solution in the presence of stirring (see Table I).

The Fig. 5 also shows the effect of outer Ca^{2+}. Increase of the outer Ca^{2+} concentration decreases the leakage of Na^+ vesicles induced by butanol approaching, around 40 mM, to the amount leaked for Ca^{2+} vesicles in Ca^{2+} solutions.

The effect of butanol on the leakage of Ca^{2+} vesicles (Fig. 5B) is much less significant. In addition, the presence of Ca^{2+} outside reverts almost completely the leakage (Table II).

Discussion

The analysis of the results indicates that immediately after the onset of stirring, leakage of carboxyfluorescein decreases in the following order: vesicles containing Na^+ in the presence of hexadecanol upon addition of Ca^{2+} in the outer solution > vesicles containing Ca^{2+} in the presence of hexadecanol in the absence of Ca^{2+} outside > vesicles containing Ca^{2+} in the presence of Ca^{2+} outside with or without hexadecanol (Figs. 2A and 4B).

A list of the specific rate constants and permeabilities is given in Table I.

The first two cases suggest that hexadecanol enhances leakage upon stirring when Ca^{2+} is in contact either with the outer or with the inner interface of the vesicle bilayer. As judging by the permeability values of Table I Ca^{2+} outside seems to be more effective than inner Ca^{2+}.

The last two cases denote that the presence of hexadecanol does not promote further leakage than that induced by Ca^{2+} present inside and outside the vesicle. All the other cases assayed did not show significant leakage upon stirring.

In the absence of stirring, vesicles also leak

when butanol is present at a concentration of 0.1 M. In these cases, Na^+-containing vesicles are much more leaky than Ca^{2+}-containing vesicles. Furthermore, the increase of Ca^{2+} in the outer solution inhibits the butanol effect (Fig. 5A). Table II shows the corresponding permeability values. The leakage induced by butanol in Na^+ containing vesicles in the absence of Ca^{2+} outside is higher than that found stirring Ca^{2+}-containing vesicles in the presence of hexadecanol/Ca^{2+} in the outer solution (see Tables I and II).

The increase of Ca^{2+} concentration up to 20 mM in the outer solution decreases the leakage of Na^+ vesicles in the presence of butanol to the level found with Ca^{2+} vesicles in the presence of Ca^{2+} or Ca^{2+} hexadecanol in the outer solution and stirring.

At 40 mM Ca^{2+}, Na^+-vesicles in the presence of butanol give the same permeability values as that obtained stirring Na^+ vesicles with Na^+ or Ca^{2+} in the outer solution.

From these results it can be inferred that there are at least two types of permeation in sonicated vesicles. One of them is the leakage induced by the onset of the stirring and the other one by molecules which are able to permeate the bilayer.

TABLE II

PERMEABILITY CONSTANTS OF Na^+ AND Ca^{2+} VESICLES INDUCED BY 0.1 M BUTANOL IN THE ABSENCE OF STIRRING

All samples contained 0.1 M butanol except the controls. The dispersing solution was 0.1 M NaCl in all cases. Permeability values were calculated from the kinetic date of Fig. 5 as described in Materials and Methods.

Inner media (M)	Ca^{2+} in the outer media (M)	P (10^{-11} cm/s)
0.1 M Na^+ (control)	0	0.071
0.1 M Na^+	0	19.35
0.1 M Na^+	0.001	19.35
0.1 M Na^+	0.020	16.12
0.1 M Na^+	0.040	11.8
0.05 M Ca^{2+} (control)	0	1.54
0.05 M Ca^{2+}	0	4.60
0.05 M Ca^{2+}	0.001	4.60
0.05 M Ca^{2+}	0.020	6.37
0.05 M Ca^{2+}	0.040	3.86
0.05 M Ca^{2+}	0.080	2.31

The leakage by stirring appears to be enhanced by membrane rigidity inducing factors such as Ca^{2+} and hexadecanol. It is generally faster than that induced by butanol and it may be related to cracks breaking-through the bilayer. These cracks may appear in rigid membranes of vesicles of large size by the deformation imposed by the vortexing and stirring. In this respect, Ca^{2+}-containing vesicles have a tighter membrane than Na^+ vesicles as shown by determinations of the anisotropy parameter with diphenylhexatriene (Bakás and Disalvo, to be published).

In addition, as shown in Materials and Methods, vesicles containing 0.05 M $CaCl_2$ are larger than those filled with 0.1 M NaCl. In fact, vesicle size is a function of the compound and concentrations present in the solution during the sonication [19]. Also, vesicle size has been found to increase when Ca^{2+} concentration increases (Disalvo, Bakás and Ohki, to be published).

When Ca^{2+} is inside the vesicle, it increases the rigidity of the bilayer inducing the Ca^{2+} adsorption on the external interface [20]. Thus, Ca^{2+} at both sides of the bilayer makes the vesicle more fragile than when it is in contact with Na^+. This is similar to the findings reported with saturated phosphatidylcholines or with acidic phospholipids [15,23,26].

In the case of Na^+ vesicles, the rigidity of the bilayer may be increased by hexadecanol and in consequence Ca^{2+} adsorption on the outer interface is enhanced. Comparison of the results of Figs. 1 and 4 indicates that hexadecanol promotes higher leakage than Ca^{2+} outside. This may be due to the fact that hexadecanol penetrates the bilayer meanwhile Ca^{2+} affects the hydrocarbon chain rigidity by interactions at the interface [27].

When Ca^{2+} is in contact with the inner face of the vesicle bilayer the addition of hexadecanol in the absence of outer Ca^{2+} promotes significant leakage. This also can be explained in terms of the penetration of hexadecanol in the bilayer affecting directly the hydrocarbon chain region. Another situation that we must discuss is the negligible effect of hexadecanol when added to Ca^{2+} vesicles in the presence of Ca^{2+} outside.

This might be due to a hindrance for the incorporation of hexadecanol into the bilayer by Ca^{2+} adsorbed on the outer interface of the vesicle (see

Materials and Methods). Another possibility is that hexadecanol can not increase further the rigidity induced on the bilayer by the action of the simultaneous presence of Ca^{2+} at both sides. However, this last situation is unlikely in the light of the results obtained with Na^+ vesicles with Ca^{2+} and hexadecanol outside (first line Table I and Fig. 4A). However, a tighter adsorption of Ca^{2+} in the external surface of Ca^{2+} vesicle in comparison to those containing Na^+ cannot be discarded.

The effect of hexadecanol, and also of butanol, as will be discussed later, seems to be related to the properties conferred to the bilayer by the Ca^{2+} distribution at each side. It is clearly seen that hexadecanol promotes the highest leakage in Na^+ containing vesicles, whose membrane are likely more fluid than those of Ca^{2+} vesicles, when Ca^{2+} is outside. In the case of Ca^{2+} vesicles the outer monolayer may be more fluid than the inner one because of the curvature [17,18]. Thus, it is reasonable that hexadecanol effect is lower on vesicles in which only the outer monolayer is fluid.

Changes in the stability of DPPC vesicles at 23°C by TEMPO C_{16} derivates have been reported [6].

In contrast, it is interesting to observe that butanol effect is completely opposed to that of hexadecanol. It induces the highest leakage in Na^+ vesicles in the absence of Ca^{2+} in the external solution, meanwhile it is negligible in Ca^{2+} vesicles. In addition, the effect on Na^+ vesicles almost disappears when Ca^{2+} is increased in the outer solution (see Table II).

The assays with butanol can be interpreted in terms of another type of permeability. Butanol can permeate the bilayer as a consequence of its partition coefficient and the direction of the concentration-gradient [24]. The penetration of butanol can expand the bilayer inducing the liberation. This in turn can be affected by the presence of Ca^{2+} outside either by a tightening of the bilayer or by a hindrance of the adsorption at the vesicle interface.

This influence of Ca^{2+} on butanol induction can be interpreted in the same line of reasoning used for hexadecanol results. In expanded bilayers (Na^+ vesicles) both hexadecanol and butanol can penetrate. In the first case, hexadecanol rigidizes the bilayers and hence Ca^{2+} may adsorb afterwards on the outer interface. The rigidity increase would produce leakage by membrane cracks upon stirring.

In the second case, butanol can penetrate fluid membranes as those of Na^+ vesicles and promotes a leakage of the vesicle content by membrane expansion.

On tighter membranes, such as those of Ca^{2+} vesicles, hexadecanol can penetrate in a certain extent (probably only the outer monolayer) and butanol cannot expand the bilayer as is suggested by the absence of leakage.

When Ca^{2+} is in the outer media hexadecanol does not enhance leakage and butanol effect is completely eliminated.

Finally, it must be pointed out that the permeability values of Tables I and II are in the order of those reported by Marsh et al. [23] for the TEMPO choline permeability in dimyristoylphosphatidylcholine vesicles.

Coincidentally, egg yolk PC vesicles prepared in buffer phosphate are much less leaky than DPPC vesicles prepared at the phase transition in the same conditions. The main difference between egg PC and DPPC has been ascribed to the number of pore creation sites, the number being much greater in DPPC than in egg PC [25].

In addition, it has been suggested that swelling of dimyristoylphosphatidylcholine vesicles induced by butanol in the fluid state is caused by perturbation of the membrane/water interface, weakening the membrane structure and water transport [26]. During the experiments here reported egg PC bilayers are in the fluid state. However, Ca^{2+} inside and outside vesicles confer to the bilayer permeability properties similar to those of saturated PC at a very few degrees above the transition temperature.

These results put into relevance that the permeability mechanisms in lipid bilayers could be related to their mechanical rigidity. In addition, it is interesting to note that those properties are a function both of the aqueous solution in which they are prepared or in which they are dispersed. In this regard, depending on the type of aqueous solute present at both sides of the vesicle bilayer the manipulation and stirring of liposomal dispersions can affect the amount of trapped solutes.

These conclusions would be of interest in the use of liposomes for basic and applied purposes.

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