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Properties of gel phase lipid-trehalose bilayers upon rehydration

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When dipalmitoylphosphatidylcholine bilayers dried under vacuum in different concentrations of trehalose are rehydrated in buffer without the sugar they show different physicochemical properties in the gel state in comparison to the normal gel state. Dry DPPC/trehalose mixtures are readily dispersed in buffer below the phase transition showing by electron microscopy a morphology similar to liposomes prepared by dispersing the lipids in buffer above the phase transition temperature. In these conditions, an increase in the peak at 570 nm of merocyanine after the dehydration-rehydration process in the presence of the sugar is observed and the water permeation increases to values comparable to those found in the fluid state as indicated by the activation energy values and the osmotic volume.The trehalose-dried liposomes rehydrated in buffer show a similar osmotic response to hypertonic gradient as DPPC liposomes without sugar near the phase transition temperature. In accordance with this behavior the trehalose-dried liposomes are lysed below the phase transition temperature by lysoderivatives. These modifications of the gel state of hydrated phospholipids by trehalose can only be achieved if a drastic dehydration is performed in the presence of the sugar. After rehydration the changes in the gel state can be detected after dyalizing the rehydrated membranes in media without trehalose during at least 24 h. These results suggest that trehalose is still intercalated between the phospholipids after restoring water to the dried liposomes either at temperatures below or above the phase transition.

Introduction

Trehalose is a dimer of glucose with the ability to preserve structural and functional properties of different types of cells, natural and synthetic lipid membranes [1–5]. This carbohydrate can maintain the gel-liquid crystalline phase transition of dry dipalmitoylphosphatidylcholine (DPPC) bilayers at a temperature near that corresponding to fully hydrated phospholipids [6].

The reason for which the main phase transition temperature of dry DPPC membranes is maintained around 42–43°C has been related to the ability of trehalose to replace water at the bilayer interface by intercalating between the phospholipid headgroups. Dry-trehalose stabilized DPPC bilayers reveals a first-order phase transition from the L_κ to the L_λ phase at temperatures similar to the L_β-P_β-L_α transition of hydrated lipid bilayers. The L_κ phase shows a typical X-ray diffraction pattern and the L_λ-phase diagram patterns indicate disordered hydrocarbon chains [7]. In contrast, the headgroup and interfacial regions show a reduced mobility, indicating that the sugar interacts extensively with the hydrophilic regions of the lipids [5–7].

Alterations in the membrane surface in the dry state have also been observed when carbohydrates are added as a derivative that incorporates to the bilayer. In this case, the sugar mimics the effect of water in the sense that it promotes an expansion of the lipid lattice normally associated with lipids passing from the gel to the liquid-crystalline state [8].

In excess water, the effect of trehalose on the membrane properties are less clearly understood. Trehalose is one of the most effective sugars to expand phospholipid monolayers in an air/water interface [9]. However, these effects are controversial due to the possible presence of surface-active impurities [10].

This sugar is also able to inhibit to a great extent the leakage of trapped solutes from liposomes during a rapid shift through the thermotropic phase transition [11]. The sugar effects on the thermotropic phase transition seem to depend on the lipid composition and are different in unilamellar or multilamellar vesicles [12].

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When the bilayer is in contact with an aqueous solution, H-bonding compounds such as sugars dissolved in the aqueous bulk phase are excluded from the phospholipid bilayer [16]. This exclusion is in part due to the presence of non-solvent water immediately adjacent to the polar headgroups. In consequence, their permeability rates are very low in comparison to other polar solutes such as polyalcohols [15–17].

Therefore, trehalose would have access to the headgroup region when the total or a partial amount of bound water is displaced. Since cells which lose activity when dried and rehydrated maintain almost full biological activity when they are dehydrated in the presence of trehalose [1,2,4], it seems reasonable that the interaction of trehalose with the headgroup region can be achieved when the total or a partial amount of the bound water is displaced.

However, as membrane functions are performed after rehydration, trehalose stabilization of membranes in the dry state is only part of the process. In order to understand the whole stabilization process it is of importance to know if the dehydration in trehalose produces modifications in the properties of rehydrated membranes.

The intercalation of the sugar and its effects on the physicochemical properties of dry membranes is achieved when the sugar is in excess with respect to the lipids [6,7]. Under these conditions, the properties of the bilayer show a number of similarities with hydrated membranes although there are also significant differences.

In this respect, it is not clearly established whether trehalose intercalated in the phospholipid bilayer in the absence of water can be totally or partially displaced from the bilayer when water is restored to the system.

A question that still remains unanswered is: What are the properties of the bilayer dried in the presence of trehalose upon rehydration?. A possibility is that, if trehalose is not displaced from the bilayer upon rehydration, the bilayer properties achieved by the intercalation in the dry state would be preserved upon hydration. The presence of trehalose in the lipid membrane after rehydration should be more noticeable when the bilayer is below the gel-liquid crystalline transition temperature, where the lipids which trehalose would be attached to, would not be able to pack as in a normal solid state. Therefore, the properties of the bilayers in the gel state of liposomes dehydrated in trehalose would be different after rehydration than those in which the dehydration-rehydration has been done in the absence of the sugar.

With this goal, we have studied the surface properties, the water permeability and the effect of lysophosphatidylcholine of liposomes prepared in different trehalose concentrations dehydrated under vacuum and subsequently rehydrated below or above the phase transition in buffer without the sugar. A comparison has been made between liposomes dehydrated under vacuum and liposomes incubated during long periods of time in trehalose solutions and as a function of the time of incubation in media without the sugar after the dehydration-rehydration process.

This study has been performed using the liposomes resulting from the resuspension in buffer of the dry DPPC/trehalose mixtures which have been extensively characterized by differential calorimetry, X-ray diffraction and infrared spectroscopy [5–7,13]. The properties of the dried membranes after rehydration have been compared with those corresponding to multilamellar system whose permeability and osmotic properties are well documented in literature [14,15,22–24].

Materials and Methods

(1) Materials

Dipalmitoylphosphatidylcholine (DPPC) and monomyristoyl-phosphatidylcholine were obtained from Avanti Polar Lipids (Birmingham, AL). A single spot was observed by thin-layer chromatography under I_2 vapor. Therefore they were used without further purification.

Lysoderivative solution showed no traces of phospholipases. L-Monomyristoylphosphatidylcholine was used because, as reported previously, it has no effect on DPPC liposomes in the gel state. Therefore, the effects observed can be ascribed to the presence of trehalose.

Trehalose purchased from Sigma (St. Louis, MO) and Fluka (Switzerland) were recrystallized from hot solutions of 80% ethanol. Activated charcoal was added to the hot sugar solutions before filtration for removing surfactant impurities.

Octadecyl rhodamine (O-Rh), merocyanine 540 (MC) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were from Molecular Probes (Eugene, OR). The purity of MC was checked by HPLC. All other chemicals were of analytical grade and used as received. The solutions were prepared either in 10 mM Tris-HCl buffer or in 1 mM Hepes (pH 7.4) using water twice distilled in a standard milli Q equipment.

(2) Preparation of dehydrated-rehydrated liposomes (D / R liposomes)

Multilamellar liposomes were prepared by dispersing in aqueous solutions of different concentrations of trehalose a dry DPPC film above the phase transition temperature. The film was obtained by evaporation of an aliquot of a lipid solution in chloroform. In the dequenching experiments the chloroform solution also contained octadecyl rhodamine in a 8% (w/w) dye/lipid ratio. At this ratio, O-Rh was self quenched.

(3) Liposome dehydration / rehydration procedure (D / R trehalose liposomes)

A measured volume of the liposome dispersions prepared in different sugar concentrations were dried either above (50°C) or below (25°C) the phase transition temperature under vacuum until a dry powder was obtained. Under these conditions, DPPC shows the phase transition temperature around 41–42°C [13]. IR spectra indicated that under these conditions the dihydrated phosphatidylcholine was obtained [5,13]. The powder obtained was rehydrated at 25°C by adding an equivalent volume of buffer without trehalose.

Aliquots of these dispersions were then added to a temperature-controlled spectrophotometer cuvette and used for electron microscopy.

Liposomes dried and rehydrated in buffer without trehalose following the same procedure were used as a control for the different assays described below.

(4) Surface properties determined by merocyanine spectra

The merocyanine spectra were obtained in the presence of liposomes in different conditions using a 1 : 100 dye/lipid ratio. An aliquot of the same liposome dispersion at the same concentration was used as reference to eliminate turbidity contributions. Merocyanine was added from a stock solution after rehydration of the dehydrated liposomes to reach a final concentration of $1 \cdot 10^{-5}$ M [25,26].

(5) Anisotropy measurements

The effect of trehalose on the membrane structure under the different conditions tested in this work was determined by fluorescence anisotropy using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe [27]. Calculations were done by means of the relation

$$r = (I_{\perp} - I_{\parallel}G)/(I_{\perp} + 2I_{\parallel}G) \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the emission at 444 nm obtained with the analyzer parallel or perpendicular, respectively, to the direction of polarization of the excitation beam at 355 nm. An aliquot of a solution of DPH in tetrahydrofuran in order to reach a 1 : 200 probe/lipid molar ratio was added to the samples after each treatment and incubated in darkness during 1 h. In all experiments, corrections for light scattering blank were made measuring the excitation/emission ratio in the absence of the fluorophore. $I_{\perp}$ and $I_{\parallel}$ values were corrected with the phototube sensitivity by the geometrical factor (G).

(6) Osmotic response of liposomes

The osmotic volume changes can be determined in a relative way from turbidity measurements of a dispersion equilibrated at various osmolar concentrations. It appears that there is a linear relationship between the pellet volume and the inverse of absorbance at 450 nm [22–24,27]. The plot of $1/A$ against the reciprocal of the non permeant concentration is linear if the liposomes behave as ideal osmometers obeying the Boyle-van 't Hoff law:

$$1/A = (1/A_0)(1/C) + (1/A_{\infty}) \quad (2)$$

in which $1/A$ is proportional to the equilibrated liposomal volume, $1/A_0$ is proportional to the osmotic volume determined by the buffer volume which is enclosed during the liposome formation and $1/A_{\infty}$ is proportional to the osmotic-dead-space which can be defined as the equilibrium volume in infinite high impermeant concentration.

Similar aliquots of liposomes rehydrated in buffer after its dehydration in the presence of different trehalose concentration were allow to reach the osmotic equilibrium (normally 7 h) at different temperatures.

(7) Water permeability

The rate of volume change can be obtained according to the following relationship [22,24,27]:

$$dV/dt = k' d(1/A)/dt \quad (3)$$

in which A is the extinction coefficient measured at 450 nm and k' is a proportionality factor.

Osmotic water outflux across the bilayer of liposomes rehydrated in buffer after its dehydration in trehalose was induced by applying a non permeant gradient over the membrane. For this purpose liposomes containing 10^{-4} M were dispersed in 0.4 M trehalose solutions. The relative changes in the extinction coefficient as a function of time were obtained immediately after mixing the impermeant solution with the liposomes in a stop-flow spectrophotometer. The mixing time was between 0.2 and 0.4 s for the different assays performed. For a given preparation, mixing at a same temperature was repeated at least ten times. These values were averaged with those obtained in the same conditions with three different preparations. The standard deviation of the measurements was around 2%.

The rate constant of the volume changes were calculated from the logarithmic plot of the relative changes in the extinction coefficient against time. The activation energies were calculated from Arrhenius plots of these rate constant vs. the inverse of temperature taking five temperatures above and five temperatures below the phase transition. Control experiments were performed under different conditions to compare with results reported in literature. Due to the high dispersion, activation energies can not be determined at the phase transition temperature.

Since relative changes are taken, the values of the activation energies obtained from the slopes are independent of the heterogeneity in the liposome size [23].

(8) Determination of the lytic effect and the critical lytic concentration

It is well known that lysoPC–bilayer interaction produces a reorganization of the lipid membrane causing a bilayer-micelle transition [28,29].

The effectiveness of the detergent action of lysoPC has been found to be the highest when the bilayer is at the gel-liquid crystalline transition. We have used this property to determine the membrane features of trehalose-dried liposomes after rehydration.

The lysoderivative solution was heated to 80°C to eliminate traces of phospholipase before its use. L-Monomyristoylphosphatidylcholine was used because as reported previously it has no effect in its monomeric form on DPPC liposomes in the gel state [29]. Therefore, the effects observed can be ascribed to the presence of trehalose.

The lysis was measured by the decrease of turbidity upon titration of a liposome dispersion with a lysoderivative solution and correlated with the increase in fluorescence obtained in the dequenching of octadecyl rhodamine. The titrations were done at 25°C in order to maintain the lipids in the gel state except otherwise stated.

Turbidity was monitored in a double-beam spectrophotometer in which temperature was controlled within ± 0.2°C by a thermostatic bath connected to the sample cuvette-holder The turbidity decrease produced by the lysolipid was related to the fraction of lysolipid in micelles (θ) according to the following equation

$$\theta = (A_{max} - A)/A_{max} \quad (4)$$

in which $\theta = 0$ for $A = A_{max}$ (maximal absorbance when total lipids are in liposomes) and $\theta = 1$ for which $A = 0$ (total lipids in micelles). After the addition of each aliquot of the lysoPC solution, the mixture was maintained at constant temperature until a constant absorbance value was obtained. The critical lytic concentration (c.l.c.) was calculated from the intersection of the two straight lines obtained by plotting θ vs. lysoPC concentration.

Lipid redistribution upon addition of lysoderivatives was measured for rehydrated liposomes after their dehydration in the presence or in the absence of trehalose using liposomes containing 8% octadecyl rhodamine. An aliquot of fluorophore-containing liposomes were mixed with liposomes without fluorophore which have been dehydrated-rehydrated under different conditions. The increase in the fluorescence upon the addition of lysoderivative was measured at 577 nm (excitation at 556 nm) in an Aminco-Bowman spectrofluorometer.

The critical lysoPC concentration at which lysis occurs can be determined by the break in the curve fluorescence vs. lysoderivative concentration. The c.l.c. values obtained by turbidity and by fluorescence were comparable.

(9) Electron microscopy assays

A small drop of each sample was spread on a 400 mesh EM carbon film grid. The preparation was dried at 22°C for 15–20 s. Then, two drops of 1% uranyl acetate (pH 7.2) were added and dried out in moisture conditions in a Petri dish for 10–15 min. Negative stained grids were examined in a JEM-1200 electron microscope at magnification of 15 000 and 50 000X.

Results

The liposomes in trehalose were prepared following the standard procedure, i.e., vortexing a lipid film in the presence of aqueous solution above the DPPC phase transition. After the dehydration under vacuum the dry powder composed of DPPC/trehalose mixtures can be resuspended in buffer without the sugar below the phase transition as easy as DPPC lipid above the phase transition. The electron micrographs of Fig. 1 show the morphological features of DPPC liposomes subject to dehydration-rehydration in the presence and in the absence of trehalose. DPPC liposomes prepared in 1 M trehalose and dehydrated under vacuum at 50°C looks, when resuspended in buffer without the sugar below the phase transition (Fig. 1B), like DPPC liposomes prepared above the transition temperature without trehalose (Fig. 1C). Aggregates were observed when DPPC liposomes without the sugar were resuspended in buffer below the phase transition temperature (Fig. 1A). Morphologies similar to those in Fig. 1A were obtained when the DPPC film was dispersed in trehalose solutions below the transition temperature without dehydration-rehydration. However, the morphology and the size of the particles obtained when the trehalose dried-DPPC mixture is rehydrated below the transition temperature (Fig. 1B) are comparable to those obtained when the DPPC film is dispersed above the phase transition (Fig. 1C). In addition, the number of small particles is noticeable reduced and the preparation looks more homogeneous.

The surface properties, the permeability and the susceptibility to the lyso-PC lytic action of the liposomes shown in Fig. 1B were studied in comparison to those shown in Figs. 1A and 1C.

Liposomes dried under vacuum in the presence of 0.1 M trehalose and rehydrated in buffer without the sugar (i.e., those of Fig. 1B), show a noticeable increase in the absorbance peak of merocyanine at 570 nm below the phase transition (trace 1 of Fig. 2A). This peak is not observed when merocyanine is added to a

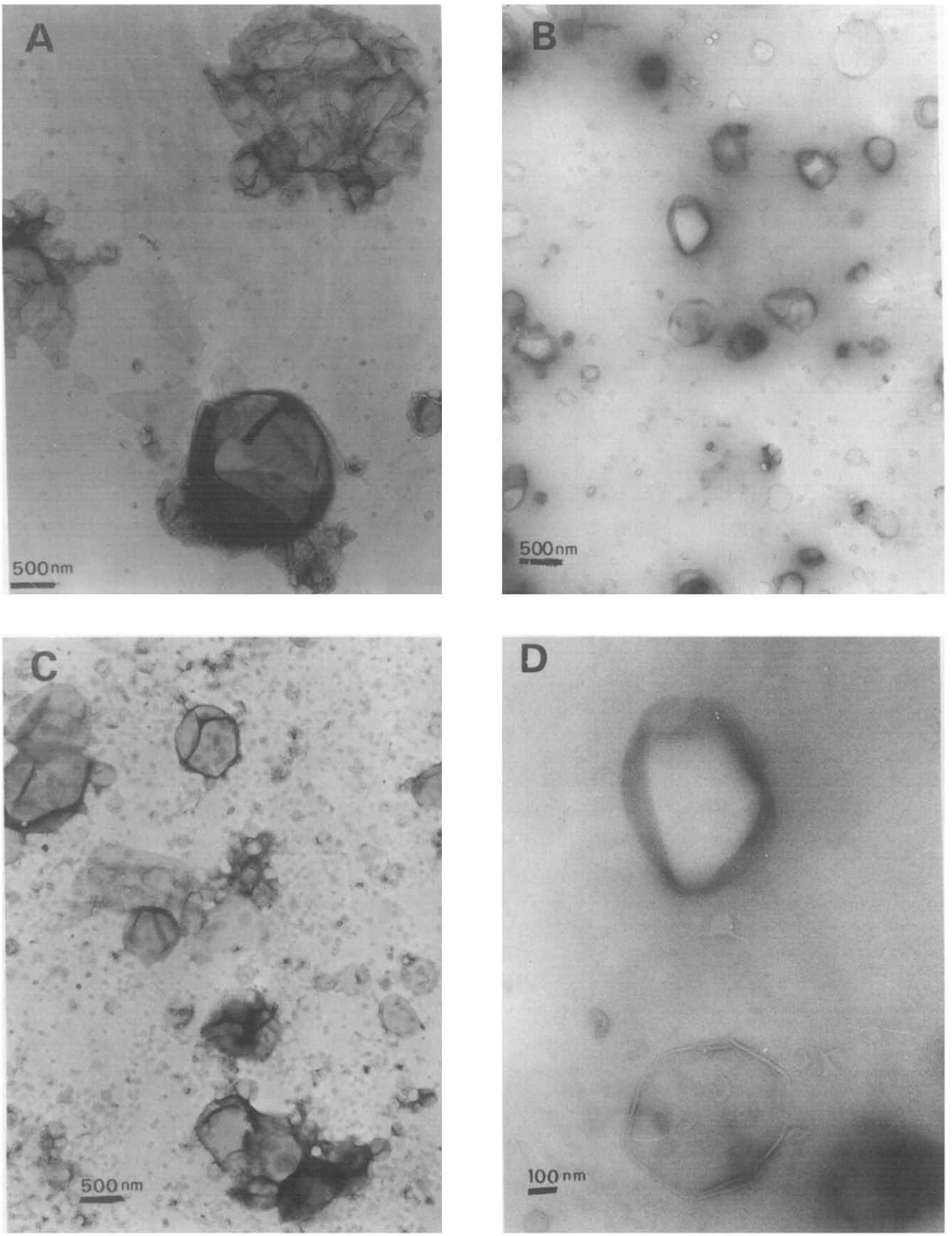

Fig. 1. Electron micrograph of trehalose-dried DPPC liposomes after rehydration. (A) DPPC liposomes prepared by resuspending the lipid film in buffer with or without trehalose below the phase transition (20°C). Magnification: 15000X. (B) DPPC film was dispersed in 1 M trehalose, dehydrated under vacuum at 50°C and resuspended in buffer without trehalose below the phase transition temperature (20°C). Magnification: 15000X. (C) DPPC film was dispersed above the phase transition temperature in buffer with or without trehalose above the phase transition temperature (45°C). Magnification: 15000X. (D) The same sample as (B) at a 50000X magnification.

suspension of DPPC liposomes below the phase transition without trehalose (Trace 2 same figure). The peak at 570 nm is characteristic of bilayers of pure DPPC in the fluid state as shown in trace 3.

Notice that the controls in Fig. 2B, indicate that liposomes prepared in 0.1 M trehalose without dehydration (trace 1), incubated at 50°C without vacuum (trace 2) or at 25°C without vacuum (trace 3) do not show, below the phase transition temperature, an increase in the peak at 570 nm.

In Fig. 3 the osmotic response of liposomes to impermeant gradients are shown. As expected, the osmotic volume of DPPC liposomes in the gel state, shown in Fig. 1A, is very low, which is a consequence of the very low permeability to water of the bilayer

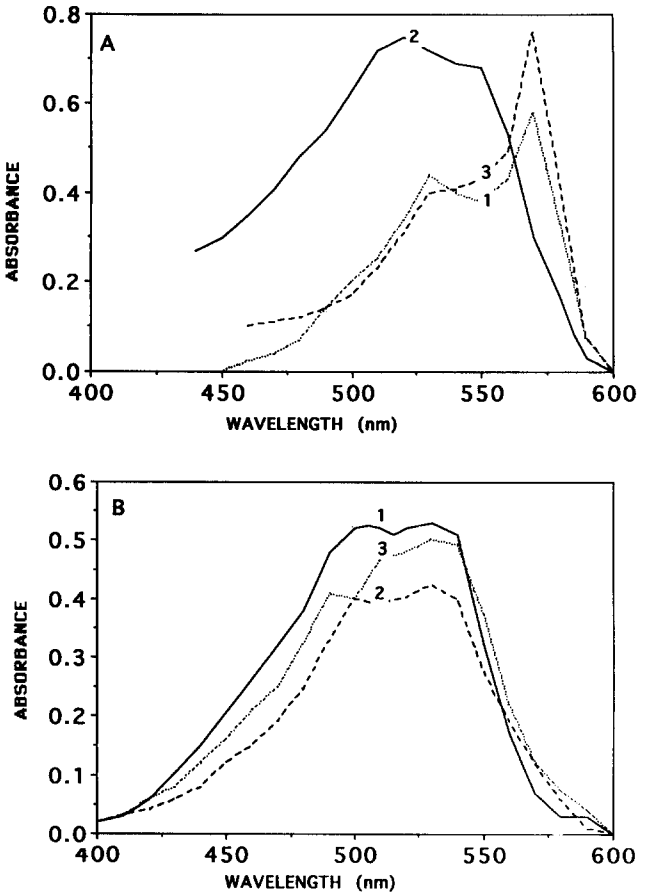

Fig. 2. Surface properties of trehalose-dried DPPC liposomes after rehydration (A) Merocyanine spectra obtained in the presence of DPPC liposomes prepared in 0.1 M trehalose, dehydrated under vacuum and rehydrated in buffer below the phase transition temperature (1); DPPC liposomes prepared in buffer without trehalose below (2) and above the phase transition temperature (3). Spectra (1) and (2) were determined at 25°C; spectra (3) were determined at 45°C. (B) Merocyanine spectra obtained with DPPC liposomes prepared in the presence of 0.1 M trehalose immediately after its preparation (1), after incubation at 50°C without vacuum for 5 h (2) and after incubation at 25°C without vacuum for 5 h (3). All spectra were obtained at 25°C. Merocyanine was added from a stock solution to the cuvette after the liposomes were equilibrated at the indicated temperatures (see Materials and Methods).

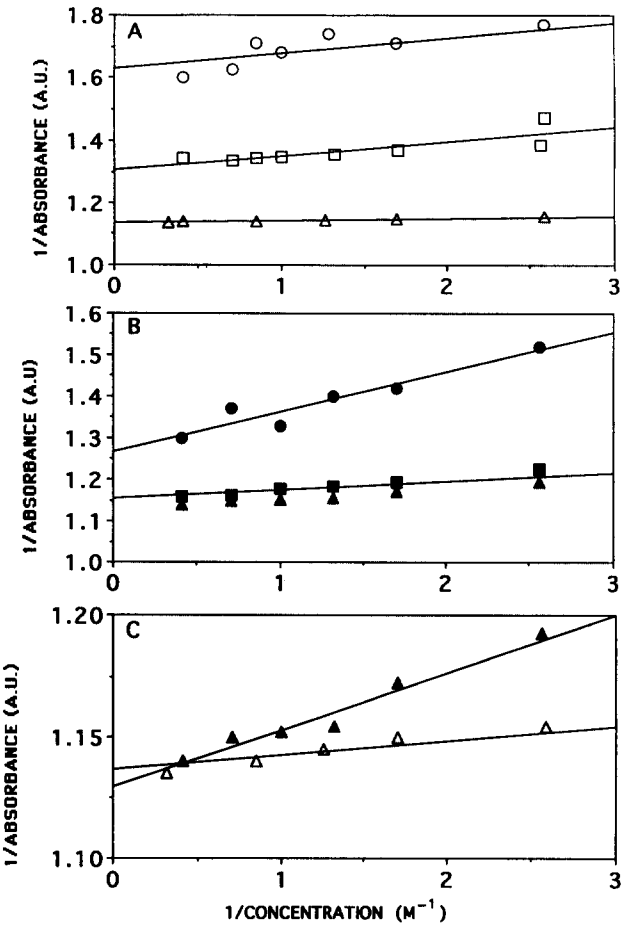

Fig. 3. Osmotic response of DPPC trehalose-dried liposomes rehydrated in buffer without sugar. (A) Osmotic volume of non-dehydrated liposomes equilibrated at different trehalose concentrations at 20°C (Δ); 37°C (□) and 45°C (○). (B) Osmotic volume of trehalose-dried liposomes rehydrated in buffer and equilibrated in trehalose solutions of different concentration at 20°C (▲); 37°C (■) and 45°C (●). (C) The same data as in (A) and (B) illustrating the differences of the osmotic volume of DPPC non-dehydrated (Δ) with DPPC trehalose-dried liposomes (▲) below the phase transition.

below the transition temperature (Fig. 3A). The osmotic volume of liposomes dehydrated-rehydrated in buffer without sugar increases near the phase transition temperature and is the highest in the fluid state.

The response of DPPC trehalose-dried liposomes rehydrated in buffer are shown in Fig. 3B. The linear plots of $1/A$ vs. $1/C$ indicate that, after the dehydration-rehydration treatment, liposomes behave as ideal osmometers (see Materials and Methods), i.e., they are sealed and semipermeable. However, it must be noticed that the osmotic volume corresponding to the trehalose-treated liposomes equilibrated below the phase transition temperature is higher than that corresponding to liposomes in the gel state without trehalose (Fig. 3C). Moreover, the value of the slope is comparable to that obtained with liposomes without trehalose near the phase transition temperature (Table I).

No variation is observed at 37°C, but above the phase transition temperature, liposomes treated with trehalose show a higher osmotic volume than those without trehalose.

The osmotic dead space (V_∞) increases with temperature both in liposomes with and without the treatment with trehalose. The values are comparable in the gel state although they are higher in liposomes without trehalose at 37°C and at 50°C. It is important to notice that, at 50°C, the osmotic dead space in trehalose liposomes is lower than those without the sugar or without the dehydration/rehydration process. In contrast, the osmotic volume is higher in the trehalose-dried liposomes.

In correspondence to the changes observed in the osmotic volume, the permeability of the bilayers to water is also affected by the dehydration-rehydration process in trehalose. The rate of volume change $(d(1/A)/dt$ can be obtained from the slopes at time zero of the transmittance vs. time traces shown in Fig. 4. The logarithmic plot of the insert shows that the water permeation follows a first-order rate process. The logarithmic plot of these rate constants versus the inverse of the absolute temperature allows to calculate the empirical activation energy.

In Table I, the values corresponding to liposomes treated with trehalose are compared with those obtained in the presence of trehalose without the dehydration step. Similar values were obtained with liposomes dehydrated in the absence of trehalose (data not shown). The high activation energy for water permeation in liposomes in the gel state is congruent with the very low osmotic volume found in Fig. 3. However, when the rate of volume change is measured at temperatures below the phase transition in liposomes dehydrated in trehalose and resuspended in buffer without the sugar a noticeable decrease in the activation energy is observed. The increase in water permeability is coincident with the increase in the osmotic volume.

TABLE I

Permeability and osmotic properties of trehalose-dried DPPC liposomes after rehydration in buffer as a function of temperature

k, osmotic volume of liposomes equilibrated in impermeant solutions according to Eqn. 2 in Materials and Methods. V_∞, osmotic-dead-space defined by Eqn. 2. E_a, activation energy for water permeation in kcal/mol as determined by the rate of turbidity change (see Fig. 4). r, anisotropy fluorescence measured with DPH. A_{570}/A_{530}, merocyanine absorbance ratio. The regression coefficient of slopes for the k values varied between 0.95 and 0.99 for temperatures below the phase transition and between 0.90 and 0.96 in the liquid-crystalline state.

Temperature (°C)		Dehydrated/ rehydrated in trehalose 0.1 M	Without dehydration
19–21	k	0.027	0.006
	V_∞	1.13	1.14
	E_a	15.4	27.4
37	k	0.020	0.020
	V_∞	1.16	1.32
	E_a	n.d	n.d
48–49	k	0.102	0.049
	V_∞	1.27	1.62
	E_a	6.3	6.0
25	r	0.233	0.284
25	A_{570}/A_{530}	1.32	0

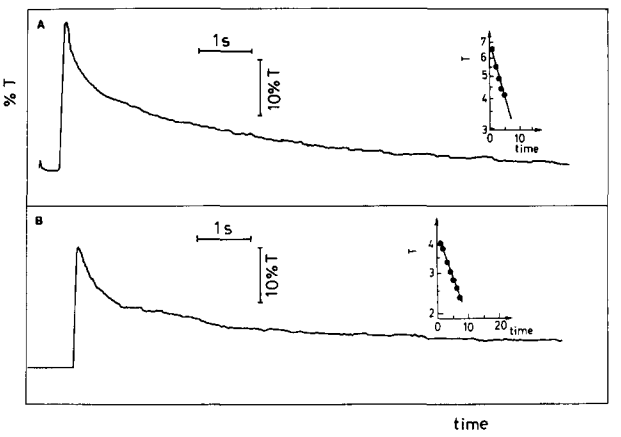

Fig. 4. Extinction coefficient of trehalose-dried DPPC liposomes rehydrated in buffer vs. time after the dispersion in hypertonic solutions of trehalose. Liposomes were mixed in a stop-flow cell below or above the phase transition temperature. (A) Trehalose dried liposomes; (B) non dehydrated liposomes. Insets: Logarithmic plot of the relative change in transmittance at time zero vs. time. The specific rate constant k calculated from the slopes is proportional to the water permeability. The scale time is given in seconds.

The values of the activation energy obtained with trehalose-treated liposomes in the gel state is intermediate between those obtained for liposomes in the gel and in the liquid-crystalline state in the absence of sugar. The decrease in the activation energy parallels a slight decrease in the fluorescence anisotropy (r) and an increase in the peak of merocyanine at 570 nm, at 25°C, with respect to liposomes without trehalose (see Fig. 2).

The osmotic response and the merocyanine peak at 570 nm indicate that the gel state of the trehalose-dried DPPC bilayer show upon rehydration some properties similar to DPPC liposomes near the phase transition temperature. In order to establish another analogy between these two systems we have studied their response to the lytic action of lysoderivatives.

It is known that the lytic action of lysoPC is produced near the phase transition temperature [28,29]. The lytic effect can be observed as a decrease in the turbidity of the liposome dispersion due to a reorganization of the lipid bilayer into micelles. The effect of lysoPCs is negligible on bilayers in the gel or in the fluid state (see Table II).

TABLE II

Critical lytic concentrations (c.l.c) for DPPC liposomes as a function of the trehalose concentration in the dehydration process

The indicated temperatures correspond to those in which the c.l.c assays were done.

Trehalose (M)	Temperature (°C)	C.l.c. (mM)	Percentage of lysis at 0.15 mM lysoPC
0	50	$>16\cdot10^{-2}$	0
0	25	$>16\cdot10^{-2}$	0
0.05	25	$>16\cdot10^{-2}$	0
0.07	25	$4.5\cdot10^{-2}$	13
0.10	25	$4.0\cdot10^{-2}$	15
0.30	25	$3.0\cdot10^{-2}$	17

In Fig. 5A it is observed that liposomes prepared in buffer, dehydrated and rehydrated in the absence of trehalose are not affected by the lysoderivative when the titration is done at 25°C. The decrease in turbidity and the extent of lysis is observed at lower concentrations of the lysoderivative when the trehalose concentration is increased.

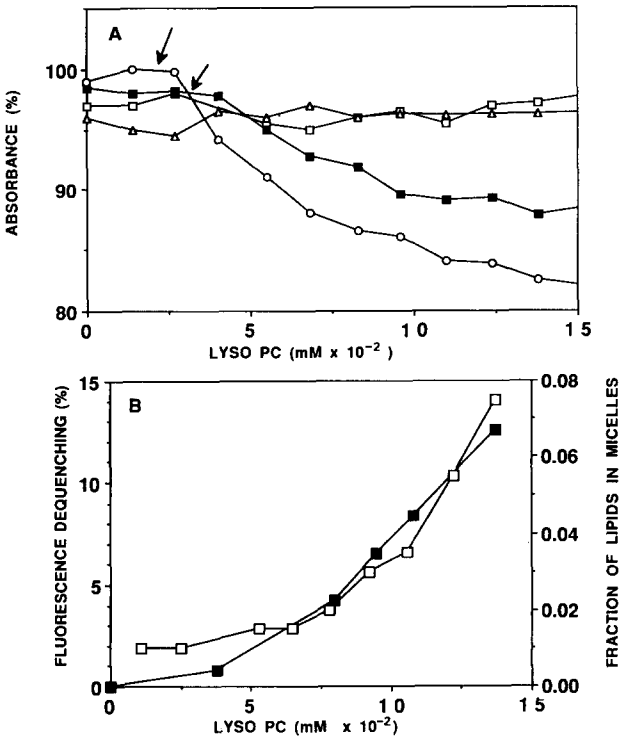

Fig. 5. Effect of lysoderivative on trehalose-dried DPPC liposomes after rehydration. (A) Effect of the lysoPC at 25°C on the turbidity of liposomes dispersion prepared in different trehalose concentrations, dehydrated and rehydrated as described previously: 0 (Δ); 0.05 M (□); 0.07 M (■) and 0.3 M (○) trehalose. Note that dehydration in the absence of trehalose lysoPC does not cause lytic effect. (B) Fraction of lipids in micelles (θ) (□) and fluorescence dequenching (■) as a function of lysoderivative concentration for trehalose-dried liposomes rehydrated in buffer. Titration was done at 25°C. The lipid concentration was 0.3 mg/ml.

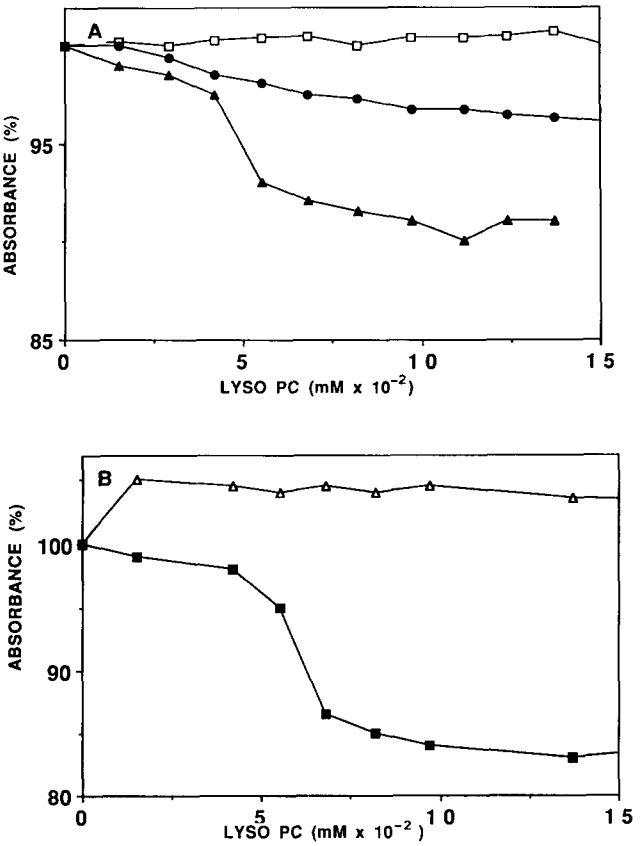

Fig. 6. Effect of trehalose on the action of lysoPC on DPPC liposomes as a function of the dehydration process. (A) DPPC liposomes dispersed in 0.1 M trehalose without dehydration (□). DPPC liposomes prepared in 0.1 M trehalose and incubated during 4 h at 50°C without vacuum (●). DPPC liposomes prepared in 0.1 M trehalose, dehydrated under vacuum at 25°C and rehydrated in buffer without sugar at 25°C (▲). All the titrations were done at 25°C. (B) DPPC liposomes prepared in 0.1 M trehalose, dehydrated and rehydrated at 50°C as described above titrated with 0.1 M lysoPC at 25°C (■) and at 50°C (△). The lipid concentration was 0.3 mg/ml.

The action of the lysoderivative promotes an increase of the fraction of lipids in micelles, as described by Eqn. 4 in Materials and Methods. The disruption of liposomes into micelles is also detected by the increase in fluorescence dequenching of octadecyl rhodamine (Fig. 5B). The break in the curves of Fig. 5B or the concentration at which the turbidity decreases in Fig. 5A determines the crytical lytic concentration, the values of which are listed in Table II for different trehalose concentrations.

The response of the liposomes to the lysoPC action is related to the presence of trehalose and to the dehydration process. In Fig. 6A it is observed that liposomes prepared in 0.1 M trehalose without being subject to the dehydration do not show changes when lysoPC is added at 25°C as it is the behavior found for DPPC liposomes in the gel state. The same preparation of liposomes incubated at 25 or 50°C during 4 h show a slight response (less than 5% of lysis) when they

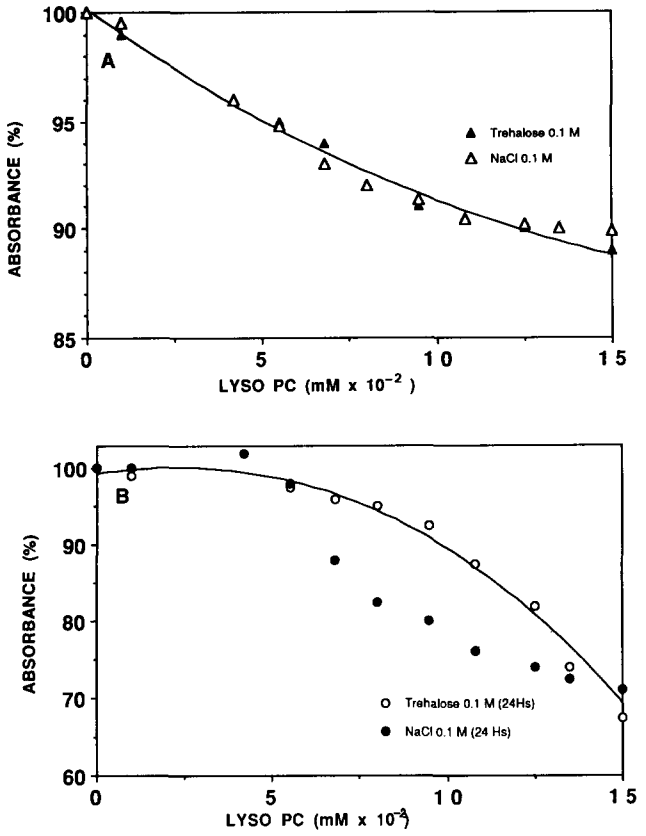

Fig. 7. Effect of trehalose in the external solution on the lysis of dehydrated-rehydrated liposomes. (A) Trehalose-dried DPPC liposomes rehydrated in buffer and titrated at 25°C with lysoPC in the presence of 0.1 M trehalose in the solution (▲). Same liposomes in which trehalose in the dispersing solution was replaced by isotonic NaCl (△). (B) Liposomes of part A after incubation at 25°C during 24 h in trehalose (○) and in NaCl (●). The lipid concentration was 0.3 mg/ml.

are titrated with the lysoPC at 25°C. However, the extent of the lysis at 25°C is increased when these liposomes are dehydrated till dryness and rehydrated at 25°C.

In part B of the Fig. 6 it is observed that liposomes prepared in 0.1 M trehalose, dehydrated under vacuum at 50°C and rehydrated at 50°C in buffer without the sugar are disrupted by the lysoPC when the titration is done at 25°C. However, when the same preparation of liposomes is titrated at 50°C no effect of the lysoderivative is observed.

In the experiments described so far the excess of trehalose present in the dried sample is dissolved in the suspending solution of the liposomes when the water is restored. However, the lytic action of lysoPC at 25°C on dehydrated-rehydrated liposomes is still observed when the trehalose of the external solution is replaced by NaCl after the rehydration. This behavior is shown in the experiments of Fig. 7. Liposomes prepared in 0.1 M trehalose were dehydrated under vacuum at 50°C and rehydrated by adding to the dry residue an equal volume of buffer without trehalose. One aliquot of this suspension was diluted in 0.1 M trehalose solution and titrated immediately. Another aliquot was diluted in isotonic NaCl in order to decrease the original concentration of trehalose in the external solution in four orders of magnitude. The titrations performed at 25°C immediately after dilution show that dehydrated-rehydrated liposomes titrated in the presence of 0.1 M trehalose in the medium superpose with those corresponding to liposome dispersions in which the external trehalose is 1000-fold lower (Fig. 7A). In addition, when liposomes of the same batches were incubated during 24 h at 25°C previous to the titration with lysoPC the lytic action is still observed in both cases (Fig. 7B).

Discussion

The rigid DPPC bilayers show aggregates in the electron micrographs (Fig. 1A) which is consistent with previous findings indicating that the adhesion of bilayers in the gel state is higher than those in the fluid state. This aggregation is not counteracted by the presence of trehalose without dehydration. However, at temperatures below T_c, DPPC/trehalose bilayers formed upon the rehydration of dry DPPC/trehalose mixtures show a morphology more similar to DPPC dispersed above the phase transition (Figs. 1B and 1C).

The increase in the peak at 570 nm, the decrease in the activation energy for water permeation and the action of the lysoderivative below the phase transition temperature indicate that the gel state of the trehalose-dried DPPC liposomes in an excess of water is different than a normal gel state of DPPC.

In spite of the increase in permeability, the fluorescence anisotropy shows a slight increase in fluidity. Therefore the enhancement of permeability cannot be totally ascribed to an increase in the degrees of freedom of the acyl chains promoting the trans-gauche isomers transitions [31,32]. In addition, wide-angle and low-angle X-ray patterns of DPPC bilayers dehydrated in the presence of 0.3 M trehalose and rehydrated in buffer are the same as those of DPPC in water (data not shown). In this condition, calorimetric determinations showed that the transition temperature was slightly displaced to higher temperatures as observed in the dry DPPC/trehalose mixtures [5,35]. In contrast, the increase in the peak at 570 nm of merocyanine indicates that the surface properties of the rehydrated DPPC/trehalose mixtures are significantly affected. According to previous results [30], trehalose has the same effect on the orientation of the phosphocholine dipoles of DPPC bilayers as the addition of small negative charges such as DPPG to the membrane. Moreover, the presence of these charges decreases the activation energy for permeation [22,24]. The activation energy of liposomes formed upon the rehydration of

dry trehalose/DPPC mixtures (15.4 kcal/mol) is comparable to that obtained with DPPC multilamellar liposomes containing 4% DPPG (14.1 kcal/mol) [24].

The similar values of permeability obtained with trehalose/DPPC liposomes and DPPG/DPPC liposomes may be related to their effects on the surface properties. It has been shown by ^{2}H-NMR that the introduction of charges as DPPG or the presence of trehalose promotes an expansion of the bilayer affecting the orientation of the phosphocholine dipoles and the water molecules in the membrane surface [30].

A possibility is that trehalose can penetrate between the phospholipid headgroups when the bilayer is in the fluid state. However, liposomes prepared in trehalose solution above the transition temperature without the dehydration step do not show changes in the merocyanine spectra and in the lytic action (Figs. 2B and 6A). Moreover, the incubation without vacuum at 25°C or at 50°C of liposomes formed in 0.1 M trehalose does not produce any effect on the 570 nm peak and the lysis.

It must be recalled than the structures observed in Fig. 1B are formed by the rehydration of dry DPPC/trehalose mixtures in buffer below the phase transition and that the surface properties observed with merocyanine and the lysis are the same after the replacement of the trehalose in the adjacent aqueous solutions by dyalisis in NaCl. In consequence, it can be thought that trehalose has been incorporated to the bilayer during dehydration of the gel state. As trehalose seems to promote an expansion of the lattice in the dry trehalose/DPPC bilayer, water molecules would have more access to the lipids when trehalose is present and in consequence the resuspension dealing to structures as those shown in Fig. 1B are easily obtained. The analysis of the permeability and lysis results indicates that trehalose is present in the bilayer even in the case trehalose in the external solution is replaced by NaCl. Moreover, trehalose-treated liposomes show a clear response to the lysoPC at 25°C. However, when the temperature is raised to 50°C no lytic action is observed. Regions with trehalose coexisting with regions without trehalose would create, at 25°C, defects on the membrane surface, as detected by the increase in the peak at 570 nm of merocyanine, into which the lysoPC can penetrate and destabilize the bilayer structure. When temperature is displaced to 50°C, both types of regions would be similar and therefore no effect of lysoPC is found.

In correlation with the response to lysoPC, which is known to act on bilayers with defects promoted by the coexistence of different phases or phase separations [28,29], the osmotic behavior of the trehalose/DPPC liposomes after rehydration is similar to that found for DPPC liposomes near the phase transition temperature.

A possible explanation of the lytic effect and the osmotic response would be that trehalose is destabilizing the gel phase and at temperatures below the DPPC phase transition a coexistence of gel and fluid phases would be found. However, since no change in the overall properties of the gel phase has been found by DSC and X-ray, several other explanations are possible. In first place, it may happen that the access of trehalose to the bilayer structure is very difficult. This is suggested by the fact that the changes in the physicochemical properties of the gel state can be observed only when a drastic treatment to displace water is applied. Even in these circumstances the number of trehalose molecules that would remain attached to the phospholipids would be very low and therefore the number of lipids contributing to the decrease in the transition temperature would not be significant.

A second possibility is that trehalose is only promoting changes at the membrane surface without affecting to a great extent the acyl chain packing. This would be supported by the observation that, in spite of an increase in permeability, the fluorescence anisotropy values are only slightly below those corresponding to a normal gel state. In this sense, the effect on permeability would be given by changes at the interface due to the appearance of defects [14,20,33] probably due to the change in orientation of the phosphocholine group as suggested previously [30].

Finally, trehalose would be able to interact both with the gel phase as well as with the fluid phase. In this case, the destabilization of the gel phase would be paralleled by a destabilization of the fluid phase and therefore maintaining the phase transition temperature nearly invariant. It is interesting to observe that trehalose promotes an increase in the osmotic volume and a decrease in the dead-osmotic space in comparison to liposomes in the fluid state without trehalose.

An explanation to this observation is that water molecules displaced by trehalose from the non-solvent layers contribute to the increase in the osmotic volume. This would be in agreement with the interpretation that trehalose changes the long-range order of the water layer at the membrane surface [30].

The osmotic dead space corresponds to the total volume occupied by the bilayer, i.e., the volume excluded by the phospholipids and the volume excluded by the non solvent water layers [16,17]. Assuming the same excluded volume for the lipids in liposomes with and without trehalose, the differences in the osmotic dead space (Table I) between liposomes after the dehydration/rehydration process corresponds to a volume of 0.36 l/mol which amounts to eight water molecules. This displacement of water would promote a decrease in the bilayer thickness and therefore an increase in permeability.

Since the process of substitution of water by trehalose would take place during the dehydration the

increase in the osmotic volume detectable in the fluid state would contribute also in the gel state to increase the separation of the lamellae and therefore the suspension of the dry DPPC/trehalose mixtures would be favored. Therefore,the resuspension of the dry DPPC/trehalose mixtures promotes new particles in which the bilayer properties below the transition temperature are different than those corresponding to pure DPPC.

In conclusion, the interaction of trehalose with lipid bilayers in the dry state promotes the formation of lipid bilayers which present different permeability, surface and stability properties than the normal phase gel state. This effect can be explained by the incorporation of the sugar into the phospholipid headgroup region even when water is restored to the system.

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