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Effect of Carbohydrates and Glycerol on the Stability and Surface Properties of Lyophilized Liposomes

S. Alonso-Romanowski, A.C. Biondi, and E.A. Disalvo

Instituto de Investigaciones Fisicoquímicas, Teóricas y Aplicadas (INIFTA), Casilla de Correo 16-Sucursal 4-(1900), La Plata, Argentina

Summary. The interaction of permeant molecules such as glycerol and urea and nonpermeants such as trehalose, sucrose, lactose and glucose with dipalmitoylphosphatidylcholine (DPPC) and egg yolk phosphatidylcholine (EPC) bilayers was studied by means of infrared spectroscopy in solid samples. The properties of the liposomes formed upon rehydration in different polyol solutions were determined by dynamic light scattering, fluorescence anisotropy, absorbance at 450 nm and merocyanine 540 spectra.

Phospholipid samples dehydrated in the presence of urea and glycerol give $\Delta\nu_{1/2}$ values for the antisymmetric stretch (P = 0 stretch) in the IR spectra; lower values are found for hydrated phospholipids.

In contrast, the same procedure in the presence of carbohydrates, gives $\Delta\nu_{1/2}$ values close or higher to those found for hydrated phospholipids, following the sequence glucose > sucrose > trehalose. This order is similar to that found in hydrated bilayers for the 570/500 nm ratio determined in the MC 540 spectra as a function of the number of OH equatorial groups of the sugars.

Liposomes lyophilized in the presence of those carbohydrates and rehydrated in buffer solution showed an increase in the 570/530 absorbance ratio in the MC spectra at temperatures below that corresponding to the gel-liquid crystalline transition. This is interpreted as an exposure of hydrophobic regions due to the carbohydrate-phospholipid interaction. In these conditions, the size at which liposomes spontaneously stabilize is a function of the type and concentration of the polyols in the aqueous solution. These changes in size are connected with packing and mechanical constraints of the bilayer for some of the sugars assayed.

Similar results to those obtained with lyophilized liposomes were found after aging liposomes in high sugar concentration solutions. A clear distinction can be made between the effect of permeant and nonpermeant molecules in regard to size, packing and hydrophobic region exposure.

Key Words phospholipid bilayers · dehydration · carbohydrates · glycerol · merocyanine spectra · liposome size · anisotropy parameter

Introduction

A wide range of polyols show protective properties on isolated membrane preparations and liposomes during freezing and drying [7]. Different cryoprotectants afford protection on the basis of different mechanisms. For instance, glycerol is widely used as a cryoprotectant, and its effectiveness is related to its ability to permeate the membrane. Other polyols such as sugars, for which bilayers are generally impermeable, afford protection on an osmolar basis, suggesting a colligative action.

Little information is available on the specific mechanism of cryoprotection of biomembranes. Some studies have reported that the interaction of carbohydrates and polyalcohols with artificial lipid membranes is due to H-bonding. In particular, the preservation of the bilayer structure and thermotropic behavior in the absence of water have been achieved using trehalose and glycerol [5, 16]. The disaccharide has been highly effective at avoiding fusion of liposomes and leakage at the gel-liquid crystalline transition temperature [7]. In contrast, glycerol is well known as a fusogenic agent and as an effective cryoprotectant [11], and it seems to induce new gel phases [16]. On the other hand, glycerol affects the pretransition in lipid bilayers [20].

In the absence of water, trehalose is able to maintain the transition temperature of dipalmitoylphosphatidylcholine (DPPC) in the range corresponding to hydrated bilayers [5].

In aqueous media, negligible effects of trehalose on the gel-liquid crystalline transition temperature have been found. However, small changes on the transition temperature induced by trehalose have been reported for curved bilayers [18]. Thus, the effect of cryoprotection cannot be exclusively attributed to colligative action. Osmotic dehydration is not sufficient to preserve dry membranes. Specific interactions between cryoprotectant and the membrane may add to colligative protection either enhancing or decreasing membrane stability [5, 19].

Lipid bilayers are practically impermeable to trehalose, sucrose and glucose [2, 4]. Glycerol per-

meates them by a mechanism involving dehydration [4, 8]. Permeant (glycerol and urea) as well as nonpermeant (trehalose, sucrose and glucose) molecules may incorporate into the bilayer structure during the freezing or lyophilization processes. It may happen that during both procedures changes in the hydration of the bilayers are involved. Therefore, when permeant or nonpermeant molecules are used to induce osmotic dehydration, these molecules not only act on a colligative basis but they can replace water at the membrane surface. Indeed, specific interactions of the polyols with phospholipids have been described [5, 19]. These interactions may have consequences on the bilayer properties after the subsequent rehydration. With this idea in mind, we have studied the effect of those compounds on the dehydrated DPPC bilayer properties by means of infrared spectroscopy. These results have been correlated with the properties of the liposomes formed spontaneously upon rehydration of films dried in the presence of those polyols.

Their size, packing, stability and surface features were determined by dynamic light scattering, and optical fluorescent probes such as merocyanine 540 and diphenylhexatriene.

The influence of those compounds on the final size of vesicles formed by sonication was also investigated.

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids and used without further purification. Egg yolk phosphatidylcholine (EPC) was obtained in the laboratory according to standard procedures [12]. Thin-layer chromatography and UV spectra in absolute ethanol were performed in order to check purity and peroxidation index, respectively.

Merocyanine 540 (MC540) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Molecular Probes Inc.

The effect of polyols on lipid bilayers has been a matter of discussion concerning problems derived from the purity of the sugars [1, 5]. In order to avoid uncertainties about the origin of the measured effects, different commercially available polyols were tested. The sugars and glycerol sources were from Sigma (analytical reagent), Merck (proanalysis), Mallinckrodt (analytical reagent), and Mann (Becton-Dickinson).

Infrared spectra of solid samples of each trademark sugar fitted well with those reported in the literature [17]. Under the resolution of the I.R. technique, impurities were below 2% (wt/wt). It was interesting to note that comparable results were obtained using sugars from the different sources named above. In all cases, the results correlated very well with structural features of the carbohydrates, such as number of equatorial OH groups.

All aqueous solutions were prepared in tridistilled water (pH 6.2 ± 0.2). When necessary, solutions were buffered at pH 7 using tris-HCl, 10 mM.

Multilamellar liposomes were prepared by dispersing in aqueous solution a dry film obtained by evaporating a chloroform/lipid solution. All preparations were carried out above the phase transition temperature.

Small unilamellar vesicles were prepared by sonication of a coarse lipid dispersion in different glycerol and sugar concentrations. The lipid dispersion was sonicated under N_2 in a bath sonicator (100 watts input) for 30 min at 20°C until clearness.

After annealing the vesicles at room temperature for 20 min, the dispersion was centrifuged for 20 min at 33,000 × g. The supernatant containing the sonicated vesicles was then transferred to the Coulter counter cuvette.

Infrared spectra were obtained in a Shimadzu 425 spectrophotometer from a solid sample prepared in KBr under vacuum by one of the following methods:

Method I: Lipids were dissolved in a methanol : benzene (2 : 1) mixture according to Crowe et al. [5]. Samples were frozen in liquid nitrogen and liophylized.

Method II: Lipids were dissolved in methanol. Then the polyols were added until complete dissolution by vortexing. Final solution was taken under vacuum until complete dryness in the presence of P_2O_5 at room temperature.

Method III: The liposomes prepared in the required polyol solution were dried, at room temperature, under vacuum until complete dehydration. Then liposomes were reformed by redispersion in buffer (Tris-HCl, 10 mM, at pH 7).

The mechanical mixtures described as control in Fig. 1, were obtained by taking samples from the container as supplied by the manufacturers without dissolving the lipids and the sugars in water or methanol.

The size determination was done in an electronic Coulter dynamic light scattering instrument. The apparatus consists of an argon ion laser source, which was focused on the sample in a glass cuvette, that maintains the temperature at 25°C. The intensity of the scattered light was detected at a 90° angle from the incident beam.

The radius of the liposomes was calculated from the correlation function. For particles in Brownian motion, the second order correlation function decays exponentially with the delay time according to the following expression:

$$g^{(2)}_{(\tau)} = B(1 + A\, e^{-\Gamma\tau}) \tag{1}$$

where B is the baseline constant, Γ is the inverse correlation time and τ the delay time. A is a constant between 0 and 1 determined by the optical system. Γ is given by $2Dq^2$ where D is the translational diffusion coefficient and q the scattering vector. D is related to the particle radius (R) by the Einstein-Stokes expression:

$$D = kT/6\pi\eta R \tag{2}$$

k = Boltzman's constant, η = viscosity of the solution, and T = absolute temperature.

The data to calculate the correlation function was accumulated after averaging 120 pulses in each sample. At least three different preparations in each condition were tested.

Refractive indices and viscosity of the solutions at different concentrations were measured in a refractometer and an Ostwald viscosimeter using water as a reference.

Absorbance measurements and spectroscopic determinations with MC 540 were performed in a 100-60 Hitachi digital double-beam spectrophotometer with thermostatized cuvette holder or in Gilford UV-Vis equipment. Phase transition temperatures were estimated measuring absorbance changes with temperature in a Beckman DU-8. In this case, 1-cm light path precision cuvettes were mounted in temperature-controlled holders,

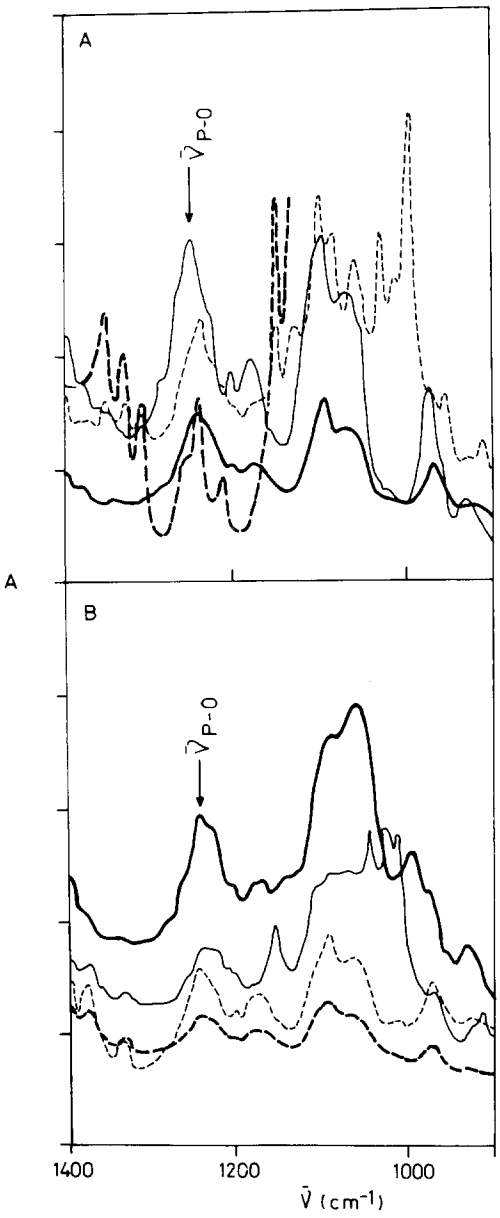

Fig. 1. IR spectra of DPPC in solid state, in the presence of (*A*) trehalose, mechanical mixture, (---); trehalose, lyophilized mixture, (▬); (▬▬) corresponds to the control experiment of pure trehalose in the absence of lipids; (——) DPPC without lyophilization. (*B*) Glycerol (▬▬); urea (---); glucose (——); sucrose (▬) lyophilized mixtures. For details *see* experimental section

both for reference and five samples, each of 200 μl total volume. Temperature was continuously varied in steps of 0.2°C at 5-min intervals and monitored according to a program which allows the system to reach the equilibrium at each temperature.

Merocyanine 540 stock solutions of 0.5 mg/ml were prepared fresh each day in water. Aliquots of these solutions were added to each sample under investigation to yield a final concentration of 10^{-7} M. Under these conditions, MC540 does not perturb lipid bilayers [14].

In all experiments, except those of Fig. 6, MC 540 was added after the preparation of the liposomes, at the moment indicated in each case.

Table 1. Values of antisymmetric stretch ν ($P = 0$) and the half-line width $\Delta\nu_{1/2}$ for dipalmitoylphosphatidylcholine (DPPC) dehydrated in the presence of trehalose (*T*) by different methods

	$\nu(P = 0)$	$\Delta\nu_{1/2}$	Condition or method[a]
DPPC (W/*l*)[b]	1246.0	46.2	Without lyophilization
DPPC (*L*)[c]	1245.0	38.5	Lyophilized method I
DPPC (*L*)	1245.2	38.05	Dehydrated method II
DPPC (*L*)	1244.8	38.5	Dehydrated method III
DPPC/T (*L*)	1240.1	42.3	Lyophilized method I
DPPC/T (*L*)	1240.5	42.3	Dehydrated method II
DPPC/T (*L*)	1240.1	42.3	Dehydrated method III
EPC (*L*)	1244.3	44.2	Dehydrated method II
EPC/T (*L*)	1240.2	38.5	Dehydrated method II

[a] *See* Materials and Methods.
[b] *W/l*: without lyophilization or dehydration.
[c] *L*: lyophilized or dehydrated samples.

In the case of Fig. 6, MC 540 was incorporated into the liposomes and gave an indication of the inner and outer interfacial bilayer properties.

Anisotropy measurements were done in a SLM-4800 spectrofluorometer using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe. For these purposes, lipid films were dispersed in solutions of different polyol concentrations. Then, an aliquot of a 2-mM DPH stock solution in tetrahydrofurane was added in order to achieve a lipid probe ratio of 130 : 1. After the DPH was added, samples were incubated in the darkness for 30 min with gentle agitation.

Results

The results of Figs. 1 and 2 show the interaction of sugar and polyalcohol molecules with the DPPC phosphate groups. The values obtained for trehalose are comparable to those reported by Crowe et al. [5] and were similar to the values we obtained using egg PC.

However, in our case, samples have been dehydrated following different methodologies. Therefore, the lipid-polyol interaction is independent of the method employed for the dehydration (Table 1). Moreover, we have compared our results with a control experiment in which lipids and sugars were mixed mechanically (i.e., lipids and sugars were mixed, without any other treatment, with KBr to form the solid sample).

From this, it can be deduced that lipid-polyol interaction arises from a reaction taking place when water is extracted from the lipids.

The values corresponding to the antisymmetric stretch ($\nu_{P=0}$) and the half-line width ($\Delta\nu_{1/2}$) for glucose, sucrose and trehalose are close to or higher than those corresponding to the nondehydrated sample (Table 2). On the contrary, samples dehydrated in the presence of glycerol and urea give

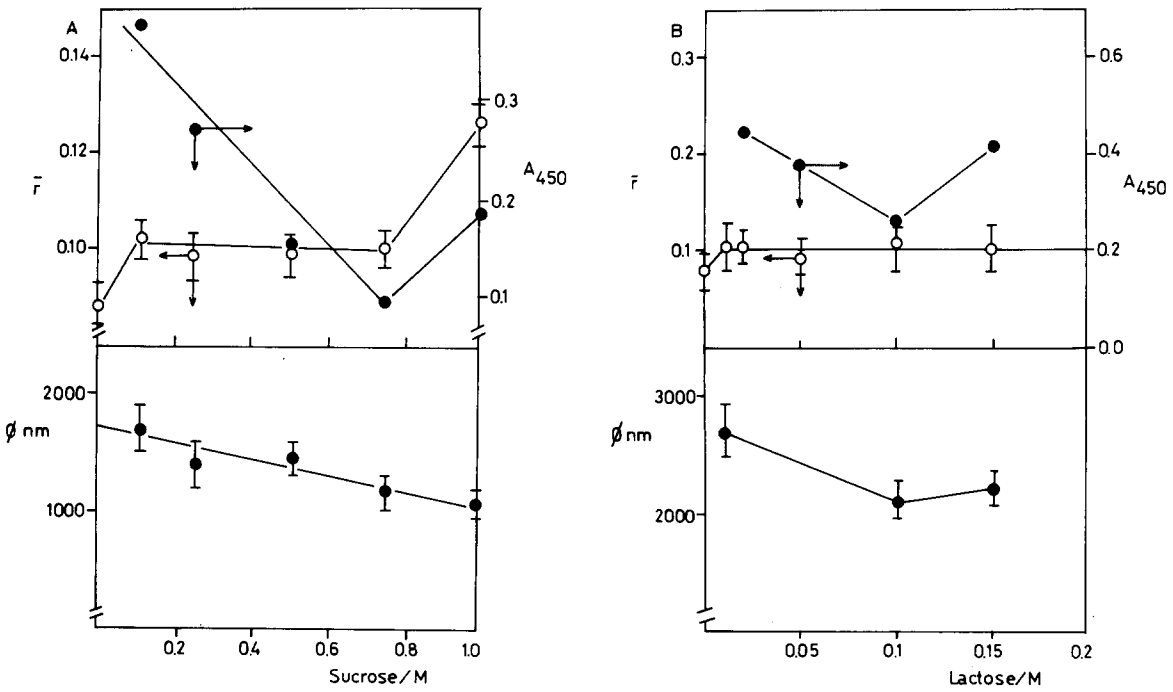

Fig. 2. Fluorescence anisotropy (r); absorbance (A 450 nm); and particle diameter (ϕ) of liposomes formed in (A) sucrose solutions and (B) lactose solutions

Table 2. Effect of carbohydrate, urea and glycerol on the antisymmetric stretch frequency ($\nu_{P=0}$) and the half-line width for DPPC

	$\nu_{P=0}(cm^{-1})$	$\Delta\nu_{1/2}(cm^{-1})$
DPPC (W/l)[a]	1246.0	46.0
DPPC (L)[b]	1245.5	38.5
DPPC + U	1242.0	40.4
DPPC + Gly	1242.5	38.5
DPPC + T	1240.0	42.3
DPPC + S	1244.0	46.2
DPPC + G	1242.0	50.0

[a] W/l: without lyophilization or dehydration; [b] L: lyophilized or dehydrated samples; U: urea; Gly: glycerol; T: trehalose; S: sucrose; G: glucose. All samples were dehydrated following Method III (*see* Materials and Methods and Table 1).

$\Delta\nu_{1/2}$ values close to that found for dehydrated lipid samples.

The polyol-phospholipid interaction also affects the properties of bilayers in excess of water.

When coarse dispersions are prepared in different sucrose and lactose concentrations, the resulting absorbance at 450 nm of the dispersion varies. This effect may be interpreted as reflecting changes in the volume of the particles according to the empirical relation between volume and the inverse of the absorbance [2] (Fig. 2*A* and *B*, upper parts).

It must be noted that the liposomes are prepared in different concentrations of carbohydrates in such a way that no osmotic gradient between the inner and the outer solution is created.

In order to corroborate this point, the size of the liposomes prepared in different sugar concentrations was determined by dynamic light scattering (Fig. 2*A* and *B*, lower part).

The concentration dependence of the turbidity and the size particle were parallel in the range 0–0.75 M for sucrose and 0–0.1 M for lactose. In these ranges, no effect on the bilayer packing was found as measured by fluorescence anisotropy (Fig. 2, open circles). However, an increase in bilayer packing was observed for sucrose concentrations above 0.8 M. In this range, the size decreased while the turbidity increased.

When liposomes were prepared in lactose concentrations above 0.1 M, no change in packing was observed and a parallel increase in size and turbidity was noticed.

DPPC liposomes prepared in glucose or glycerol solutions also gave different absorbances (Fig. 3*A*). The absorbance increased with the glucose concentration of the lipid dispersion, while the opposite was found for glycerol both below and above the phase transition temperature. It must be noted, however, that in both cases the concentration increase did not affect the anisotropy parameter (Fig. 3*B*).

The absorbance increase in glycerol solutions and the discontinuity at 0.7 M correlated with the changes observed in liposome sizes (Table 3).

Table 3. Diameter of egg phosphatidylcholine liposomes prepared in different aqueous solutions

Concentration (M)	ϕ in glucose (nm)	ϕ in glycerol (nm)
1.00	1341 ± 190; 1010 ± 172	1501 ± 354
0.75	588 ± 109; 1150 ± 137; 1210 ± 310	822 ± 210
0.50		1157 ± 127
0.25	722 ± 188; 1192 ± 167	
0.1		1200 ± 137
Buffer Tris	2178 ± 640	

Table 4. Liposome sizes and standard deviations of the extinction coefficient of egg PC liposomes prepared in different molar ratios of glucose and glycerol

Glycerol/glucose	$\sigma_{(n-1)}$	ϕ (nm)
1	0.59	1613 ± 295
0.75	—	1592 ± 81
0.70	0.66	—
0.50	0.73	—
0.30	0.78	—
0.25	—	1544 ± 239
0	1.11	1341 ± 25

Total molar concentration was 1 M. Standard deviation was determined by the equation

$$\sigma_{n-1} = \frac{\Sigma_{\chi^2} - \left(\frac{\Sigma_\chi}{n}\right)^2}{n-1}$$

for a number of samples $n = 10$.

The size determination by dynamic light scattering of the liposomes prepared in glucose was extremely irreproducible (*see* Table 3). This peculiar phenomenon was not observed with glycerol and other sugars such as sucrose and trehalose. The reproducibility was greatly improved when glucose was partially replaced by glycerol in the aqueous solution mixture. The high standard deviation obtained with liposomes prepared in 100% glucose solution was significantly reduced when the lipids were dispersed in solutions containing increasing glycerol/glucose ratios. The total molarity was 1 M in all cases. The extinction coefficient was determined in at least 10 different preparations of liposomes for each condition assayed. Values for the standard deviations and the liposome sizes obtained in glycerol/glucose aqueous mixtures are compared in Table 4. Table 5 indicates that the final size of the vesicles obtained by sonication are also dependent on the polar solute type and concentration in the aqueous solution.

The properties of the lipid-water interfaces of the liposome bilayers stabilized in aqueous solu-

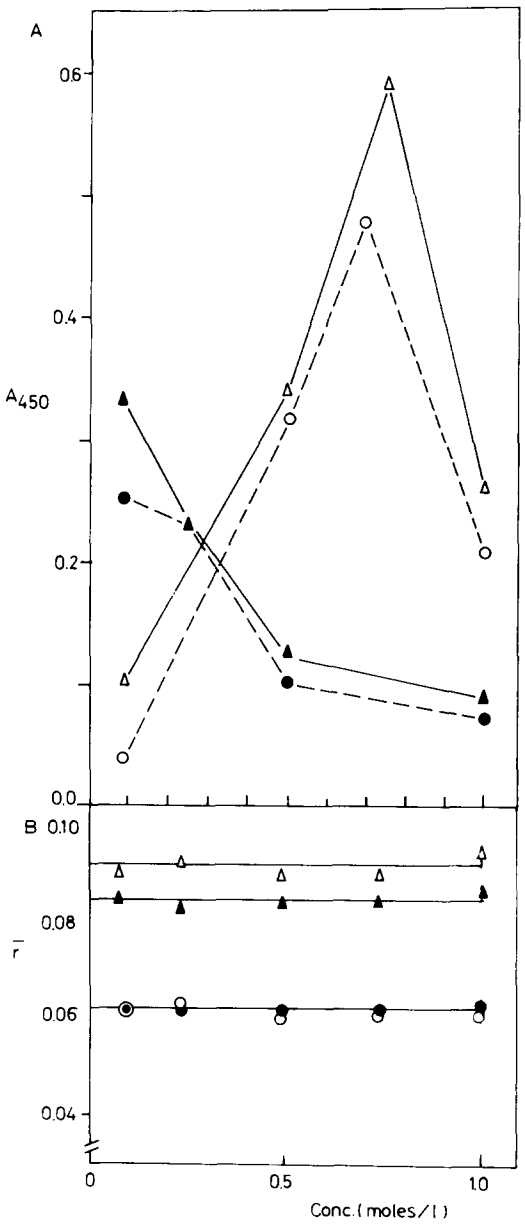

Fig. 3. Absorbance (*A*) and fluorescence anisotropy (*B*) of DPPC liposomes prepared in glycerol and glucose solutions above the phase transition temperature and measured at 52°C(●) and 26.5°C(▲) (for glucose); 52°C(○) and 26.5°C (△) (for glycerol)

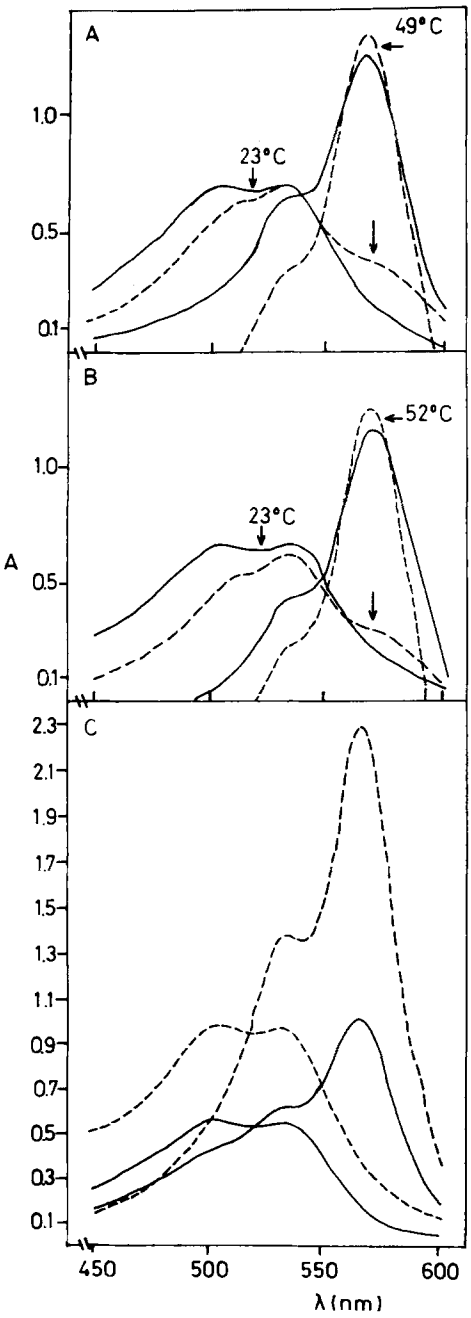

Fig. 4. Absorption spectra for MC 540/DPPC liposomes mixtures. (*A*) Liposomes prepared in buffer and lyophilized in the presence of trehalose (---) or prepared in 10^{-3} M trehalose (——); spectra was obtained for liposomes below and above the liquid crystalline gel transition temperature. (*B*) Idem (*A*), but in the presence of glucose. (*C*) Idem (*A*), but in the presence of glycerol

tions of the different polyols assayed above were studied using Merocyanine 540 as an optical probe.

With this probe, the relative magnitude of the peaks corresponding to 500 and 570 nm denotes the bilayer transition from the gel to the liquid-crystalline state [14]. The peak at 500 nm disappears at the expense of the increase of that at 570 nm. This is interpreted as an increase of the partition of MC 540

Table 5. Size of egg phosphatidylcholine vesicles (nm) prepared in different polyol solutions and glycerol/glucose ratios for a 1 M total concentration

Polyol	Diameter (nm)				
Concentration (M)	0.1	0.25	0.5	0.75	1
Glucose	—	134	—	101	101
Glucose : glycerol (75 : 25)	—	—	—	—	87
Glucose : glycerol (25 : 75)	—	—	—	—	75
Glycerol	90	—	75	—	57
Sucrose	80	—	—	122	172

Vesicles were sonicated for 1 hr, at 100 watts, and annealed for 2 hr at room temperature. Size was determined at 25°C as described in Materials and Methods.

in the hydrophobic regions of the bilayers, because it appears at the same wavelength found for MC 540 in nonpolar solvents.

The peak at 530 nm practically remains unchanged and it represents the dimer of MC in the aqueous phase [14].

The results of Fig. 4 show the spectra of MC for liposomes lyophilized in the presence of trehalose, glucose or glycerol (parts *A*, *B* and *C*, respectively) and rehydrated in a buffer solution. MC was added at this stage from a stock solution. In the cases of trehalose and glucose, a shoulder at 570 nm for liposomes in the gel state was observed. Glycerol, in the same conditions, did not promote significant changes (*see* arrows).

In addition, no effect of the hypertonic media on the MC spectra obtained without liposomes was observed.

Nonlyophilized liposomes without or at a low concentration of sugars or glycerol did not show any shoulder at 570 nm at the gel state.

Lyophilization in the presence of glycerol gave the same results as control experiments except for a displacement of the whole spectra of the lyophilized sample to higher values of absorbance. The absorbance of lyophilized liposomes in the fluid state was also greatly increased by glycerol (Fig. 4*C*).

The effect of trehalose seems to be a consequence of the lyophilization process. It must be recalled that the values of Tables 1 and 2 were obtained from solid samples subjected to this process. When liposomes were not lyophilized, no increase in the peak at 570 nm was observed even at 1 M concentrations (Fig. 5*A*). However, the shoulder was clearly defined when the liposomes were lyophilized in 1 M trehalose without aging. In the same figure it can be observed that similar effects can be obtained with nonlyophilized liposomes if they are incubated in 1 M trehalose solution at 4°C for 48 hr in the presence of MC 540. This aging also produced

a net increase of the shoulder at 570 nm at concentrations 10 times lower than 1 M trehalose. The effect observed after aging lyophilized liposomes in aqueous solution, did not depend on the presence of MC 540 during such aging. In the assays of Fig. 5*B*, MC 540 was added in the moment of the spectra determination after aging the rehydrated liposomes in 10^{-4} M trehalose for 24 and 48 hr.

There were two conditions in which the peak at 570 nm was enhanced. In one of them (Fig. 5*A*), the increase is observed after the aging of nonlyophilized liposomes in 1 M trehalose in the presence of the dye. In Fig. 5*B*, the effect was obtained for lyophilized liposomes in 1 M or 10^{-4} M trehalose. In this case, the dye was added at the moment the spectra was obtained.

Finally, the control experiment in Fig. 5*C* shows that the effect observed in Fig. 5*A* and *B* was promoted by the sugar. MC in the absence of trehalose did not affect the spectrum.

The combined effect of the sugar and MC during aging, shown in Fig. 5*A*, may be ascribed to the entrance of MC into the liposomes. If this is the case, the interfaces of the inner multilamellar liposome bilayers would be available for partitioning the probe. In order to check this possibility, we compared, in Fig. 6, the results obtained with liposomes prepared in aqueous solution of MC and glucose or glycerol with those obtained when MC was added after the liposomes were formed. That is, spectra were obtained from liposomes with MC inside and outside and from those with MC only outside. In this case, liposomes were not lyophilized.

As expected, MC in contact with the inner and the outer lipid-water interfaces showed an increase of the shoulder at 570 nm. This indicated that the effect of sugars during aging was not only due to changes in the outer interface but also affected the inner layers of the multilamellar aggregates.

The effect of aging lyophilized liposomes, after their rehydration, for 24 hr at 4°C in an aqueous solution of glucose, is similar to that found with nonlyophilized liposomes prepared in MC and 0.5 M glucose (*cf.* Fig. 6*B*, dashed curve, with full-line curve of part *A*). At this low concentration, trehalose has a greater pronounced effect (Fig. 6*C*). Note that the increase at the peak at 570 nm obtained with liposomes lyophilized in 10^{-4} M trehalose and aged, after rehydration, at 4°C for 48 hr is comparable to that obtained with 0.5 M glucose with MC inside and outside the liposome.

It is clear from these results that trehalose and glucose promote changes in the interfacial properties in contrast to the behavior of urea, glycerol, sucrose and lactose. It is not surprising that permeants such as glycerol and urea may have different effects on bilayer properties compared with sugar molecules for which membranes are practically impermeable. However, the effect of sugars is determined by the spatial molecular configuration.

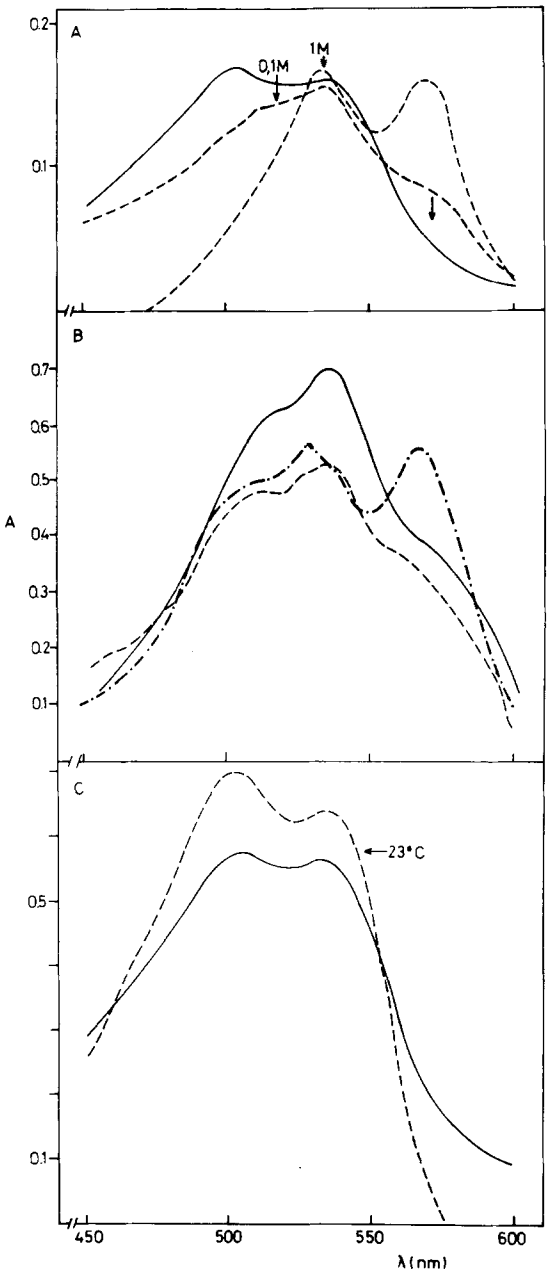

Fig. 5. Absorption spectra for MC 540-DPPC liposome mixtures. (*A*) Prepared in 1 M trehalose solution and measured immediately (——) or after aging for 48 hr, at 4°C in the presence of MC 540 (---). (*B*) Liposomes lyophilized in the presence of 10^{-4} M trehalose and aged for (——) 24 and (---) for 48 hr in the absence of MC 540 at 4°C. MC was added at the same time the spectra was run (·····) Liposomes lyophilized in 1 M trehalose. (*C*) Liposomes prepared in buffer Tris and aged with (---) and without MC 540 (——) in the solution

The magnitude of the 570/500 ratio is a function of the OH equatorial number of the sugar as shown in Fig. 7. The correlation can only be obtained with rehydrated lyophilized liposomes in the gel state or nonlyophilized in the fluid state.

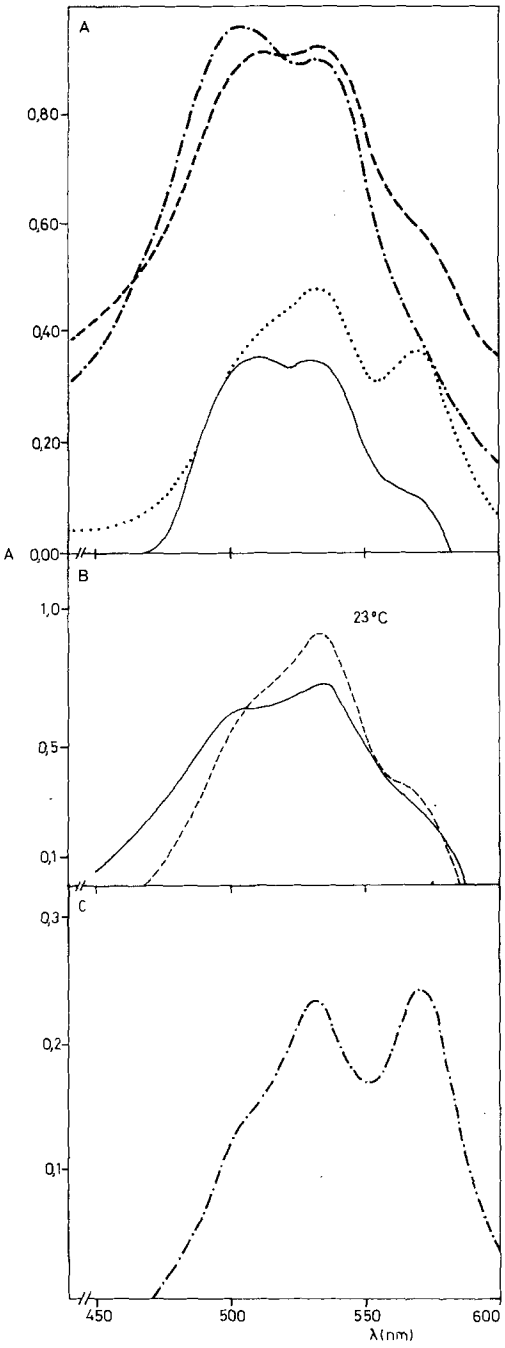

Fig. 6. Absorption spectra of MC 540-DDPC liposome mixtures in the presence of different concentrations of glycerol and glucose. (*A*) Liposomes were prepared in conditions in which MC 540 was present inside and outside the liposomes. Compare the effect of glucose with that shown in (*B*) in which MC 540 was added after the liposomes were formed and at the time the measurement was done. (·····) glycerol, 0.1 M; (---) glycerol, 0.5 M; (····) glucose, 1 M; (——) glucose, 0.5 M. All spectra were obtained at 25°C. (*B*) DPPC liposomes were prepared in 10^{-4} M glucose solutions. (---) lyophilized in the presence of glucose and aged 24 hr at 4°C; (——) samples were not lyophilized. (*C*) Liposomes lyophilized in 10^{-4} M trehalose and kept at 4°C for 48 hr in the presence of MC

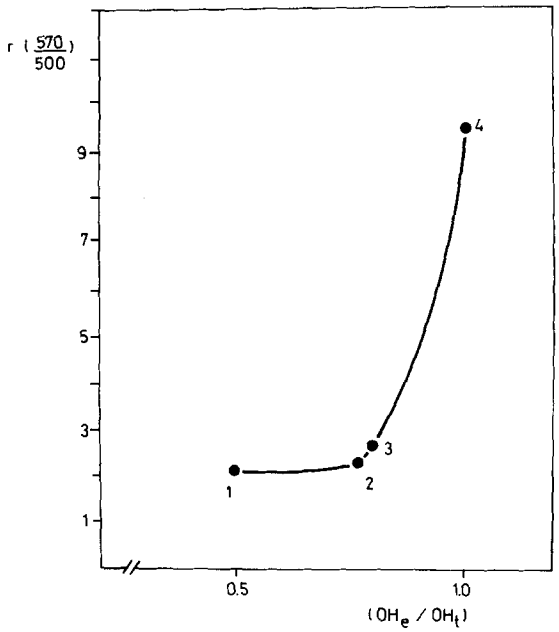

Fig. 7. Absorption ratio 570 nm/500 nm for MC 540 in the presence of liposomes at the liquid-crystalline state as a function of the fraction of equatorial OH of the different carbohydrates: (*1*) lactose, (*2*) glucose, (*3*) sucrose, and (*4*) trehalose

No correlation was found with liposomes in the gel state, which had not been previously lyophilized.

Discussion

The elimination of water in the presence of H-bonding compounds affects the polar head groups. This suggests a competition of sugars and polyalcohols by the phospholipid water sites.

A distinction can be made, according to the present results, between those compounds with the ability to permeate the bilayers in the hydrated state and the nonpermeants.

In the first case, molecules such as urea and glycerol yield values comparable to those obtained for dehydrated phospholipids (Table 2). On the other hand, molecules such as carbohydrates, which are not able to permeate lipid bilayers fully hydrated in an excess of water, yield values well above those corresponding to hydrated phospholipids. In addition, the $\Delta\nu_{1/2}$ values follow the sequence: glucose, sucrose, trehalose. This order is similar to that found in hydrated bilayers for the 570/500 ratio as a function of the OH equatorial groups (Fig. 7). It should be noted that this sequence is only found when the interface is in the

fluid state, or when liposomes in the gel state have been lyophilized.

The increase in hydrophobicity of the lipid-water interface, shown by the MC results with hydrated liposomes, is parallel to the decrease in $\Delta\nu_{1/2}$ values for bilayers in the anhydrous state, because the $\Delta\nu_{1/2}$ values are proportional to the dipolar moment given by the orientation of the phosphocholine groups with respect to the plane of the membrane. The sequence would indicate that glucose allows a closer approach of the phospholipids in comparison with sucrose and trehalose. If this situation is also taking place when the sugars are replacing water in lyophilized liposomes, it is reasonable to think that MC has more access to bilayers with trehalose, in comparison to those with glucose. This is coincident with the fact that the greatest expanding effect on phospholipid monolayers follows the order: trehalose, sucrose, glucose [6].

Accessibility of the sugars to the bilayer interface can be achieved by different ways as shown in the experimental section: when water is evacuated from liposome dispersions containing sugars or by allowing a long time contact of higher concentration sugar solutions. The lyophilization increases the effectiveness of aging. Only molecules, such as sugars, for which the lipid bilayer is known to be impermeable, appears to be able to replace water at the interface.

Permeant molecules, such as glycerol, have no significant effect even in the case of liposomes containing MC inside and outside.

As shown in Table 2, the $\Delta\nu_{1/2}$ values obtained with samples dehydrated in the presence of glycerol are approximately the same magnitude as that found for anhydrous phospholipids. That is, the magnitude of the dipolar moment at the region of the $P = 0$ group would remain unchanged in the presence or in the absence of glycerol.

This suggests that either glycerol does not incorporate to the anhydrous bilayer or, if it does, it is not able to preserve the structural parameters, which characterize the hydrated interface as is the case with sugars. According to experiments depicted in Fig. 4*C*, glycerol does not promote hydrophobic regions at the gel state when it is below 0.5 M concentration. The information obtained with MC spectra for bilayers in the excess of water below the transition temperature can be paralleled to the data reported for monolayers experiments [6]. Below the phase transition temperature, the area per molecule of DPPC is the same with and without glycerol in the subphase. Sucrose seems to be a compound with the ability to preserve the structural parameter at the values corresponding to the bilayer with water. This is suggested by IR parameters for the lipid bilayers shown in Table 2. Also, trehalose is efficient in this respect as it has been checked by DSC calorimetry [5, 19]. This replacement of water by sugars allows the bilayer to maintain regions, probably at the interface, with similar properties to the fluid state (the 570-nm peak) coexisting in a gel structure; that is, carbohydrates would tend to expand the bilayers. An alternative explanation would be that osmotic dehydration may be influencing the MC distribution. As discussed before, liposomes are prepared in such a way that no osmotic difference between the inner and outer liposome solution results. However, osmosis may be occurring between the bilayer itself and the adjacent aqueous media. This redistribution of water at each interface would allow trehalose to interact with the bilayer. Due to its size, trehalose would maintain the bilayer expanded as in the fluid state. This state would favor the entrance of MC. It should be noted, however, that the effect of sugars on the monolayer expansion has been a controversial point. On one hand, different authors have reported on specific expansion [3, 15]. However, a recent study ascribed that expansion to impurities in the carbohydrates [1].

The reproducibility of our results, obtained with a series of sugars, and the fact that they fit in a sequence as a function of sugar configuration, would discard impurities in the sample.

The irreproducibility in size and turbidity seems more likely to be a consequence of the type of sugar present in the solution in which liposomes are formed. In the case of glucose, the replacement of water seems to force the phospholipids to a less favorable conformation. As seen in Table 2, the $\Delta\nu_{1/2}$ are the highest in comparison to the other sugars. In contrast, glucose gives the lowest 570/500 ratio, suggesting a packed structure through which MC can not penetrate.

This packing seems to affect the mechanical stability of the bilayer as seen in the irreproducibility of the results in size and absorbance. The great standard deviation obtained in the size and the extinction coefficient of liposomes prepared in glucose solution (Table 3) can be ascribed to the formation of the lipid bilayer in a state in which the polar head groups are displaced from their configuration of minimum energy, as derived from the $\Delta\nu_{1/2}$ values.

In this sense, it would be expected that structures formed in the presence of glucose would be more fragile under mechanical stress. In contrast, sucrose and trehalose, which show less deviation of the $\Delta\nu_{1/2}$ from those obtained with water, form very stable and reproducible dispersions.

It must be noted that, the philosophy of this

work was to show the properties of the lipid bilayers when they stabilize spontaneously in water solutions containing H-bonding compounds. Formation of multilamellar liposomes is favored thermodynamically and the particles would be the result of the balance between the cohesive forces among lipids and the interaction with the aqueous media. Other methods, such as sonication, reverse evaporation and extrusion introduce forces to form the liposome. Anyway, the presence of H-bonding compounds during sonication influences the final size of the vesicles (Table 5).

In light of these results, it is interesting to observe that the presence of glycerol stabilizes liposome samples prepared in glucose. The permeant molecules seem to relax in part the tensions appearing in the bilayer when water is replaced by glucose. From the fact that the liposomes are more reproducible under shaking and stirring, we may argue that the increased stability is due to the replacement of glucose by glycerol in the bilayer interface. A similar phenomenon of mechanical fragility has been found in sonicated vesicles containing Ca^{2+}, a phenomenon that can be related to the dehydration effect of this ion [10].

The final size of the liposomes spontaneously formed in different aqueous solutions is a consequence of their thermodynamic stability, and this in turn is conferred by the polar group H-bonds concerted with the surrounding medium. Water, the natural stabilizing compound, can be replaced by sugars according to its spatial configuration in H-bonds in order to keep the original features.

If this is not the case, the tension created by the replacement at the head group region could promote the exposure of hydrophobic groups, with the result of an increase of the surface-free energy. At this point, the bilayer may decrease the free energy excess at least by two extreme possibilities. If permeants are present, such as glycerol, they can interact with those exposed regions (probably the fluid regions induced by glucose), penetrate and swell the bilayer relaxing the membrane tensions (*see* Table 4). In the absence of permeants, the structure would break down and reform because the mechanical stability of the structure is labile (*see* Table 4 for 100% glucose).

In conclusion, the properties of lipid bilayers are a function of the solute in the aqueous solution. In this regard, the main difference is obtained in the packing, size and interfacial properties of bilayers spontaneously stabilized in permeant and nonpermeant aqueous solution. In addition, the type of polar solute present in the solution during the sonication affects the final size of sonicated vesicles (Table 5) [*see also* 11].

As observed in Fig. 2, absorbance values frequently used as a measure of the liposome volume in permeability experiments are not a direct measurement of the size of liposomes in the whole range of concentration.

The strength of the sugar-phospholipid interaction, as measured in solid anhydrous samples (Table 2) is related to the more probable size of the liposomes formed in aqueous media. The absorbance values are a combined result between the bilayer packing and size as shown by the anisotropy parameter and dynamic light scattering, respectively.

Above 0.8 M sucrose, the absorbance increases with an increase in the anisotropy values, while size decreases. This suggests that sucrose promotes particles of smaller size by increasing the density of the bilayers. According to the preparation procedure, lipids are dispersed in the carbohydrate solution. Thus, no concentration difference between the inner and the outer solution is created. Therefore, the increased anisotropy and decreased size of the liposomes can not be ascribed to osmotic dehydration by sucrose outside the particles. However, a dehydration of the bilayer itself by the low water activity at each side of the membrane may be influencing the size and packing values [13].

Below 0.8 M sucrose and for lactose, absorbance seems to be determined by the size of the particles as can be inferred from the fact that the anisotropy values remain constant along the whole range of concentrations tested.

At these concentrations, neither glucose nor glycerol affects anisotropy values, although due to the irreproducibility in the size determinations, conclusions about the changes in absorbance can not be derived. However, it is clear that glucose affects the interfacial properties, while that is not the case for glycerol in all the different conditions assayed. This means that the bilayer fragility in glucose would be related to the redistribution of groups and bonds at the interface. The IR parameter $\Delta\nu_{1/2}$ for glucose is indicating the maximum departure with respect to that found in hydrated phospholipids. The insertion of sugar at the interface in aqueous media would create a space, thus giving the opportunity for MC to diffuse into the inner bilayers of the multilamellar liposomes. These results suggest that interfacial water and its replacement by sugars or permeant molecules such as glycerol would have to be considered on the permeability mechanism of lipid bilayers. The aqueous interfaces contribute to the permeability properties both from a thermodynamic as well as from a structural point of view [9, 11]. Hence, structural changes during permeation can be taking place.

Moreover, the changes in those properties occurring in lyophilization/rehydration processes may be of importance and may account for the optimization of cell-conservative methodologies.

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