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Effect of glycerol on the interfacial properties of dipalmitoylphosphatidylcholine liposomes as measured with merocyanine 540

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Liposomes of dipalmitoylphosphatidylcholine (DPPC) prepared in increasing glycerol / glucose ratios show an increase in the absorbance at 570 nm of merocyanine spectra at temperatures below the phase transition. Since this effect is not observed when liposomes are prepared in solutions containing solely glucose, it is attributed to specific interactions of glycerol with the membrane phase. The increase in the 570 nm absorbance is ascribed to a partial fluidification of the membrane interface and is dependent on the distribution of the dye between the inner and the outer compartments of the liposomes and on their osmotic state. The greatest differences in the absorbance ratio are obtained when merocyanine is added to the external media. In consequence, the changes in the spectra of MC are dependant on the surface state of the liposomes which can be modified by an increase of glycerol or glucose in the external media. The present results are examined in the light of the perturbations that glycerol can induce on the barrier properties of the bilayer.

Introduction

It is well known that glycerol permeates natural and artificial lipid membranes. The mechanism of its penetration would involve a dehydration step of the molecule before entering the membrane phase in relation to the number of hydrogen bonds that the molecule can concert with the aqueous phase [1,2].

Physicochemical factors such as temperature, osmotic pressure and chemical action of solutes present in the aqueous solutions can affect lipid–lipid and lipid–water interactions [4,5]. In this context, little attention has been paid to the changes that permeant molecules can produce on the membrane structure and how these changes affect the membrane permeability. Although there are evidences that glycerol, in particular, affects the phase state and the interdigitation of the lipid bilayer [6–8], permeability studies usually consider the membrane as a non-perturbed structure [4].

Classical formalisms used to calculate permeability consider the lipid membrane as a phase interposed between two aqueous compartments thick enough to make the interfaces negligible. Obviously, this is not the case in a lipid membrane and consequently lipid membrane and aqueous environment respond as a single thermodynamic entity to physicochemical factors [5]. This is to say that the overall properties of the membrane are due to the organization of the lipids in the bilayer and the lipid-water association at the interfaces.

The high glycerol concentration to built the gradient may affect the bilayer structure both by the osmotic volume contraction or by direct interactions of the glycerol with the lipid components.

In this direction subtle changes have been found by X-ray diffraction, calorimetric measurements and spin paramagnetic resonance when glycerol substitutes for water [7]. The exchange of water by glycerol affects the polar head group conformation as measured by infrared spectroscopy [9,10]. Therefore, it is expected that the conditions to which liposomes are exposed in a permeability assay, namely changes in temperature and high glycerol concentrations at one or both sides of the bilayer, may cause perturbations in the membrane regions which are relevant to determine the permeation kinetics.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; MC540, merocyanine 540.

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In addition, the activation energy from which the dehydration energy is calculated is a function of the osmotic state of the liposome [3,4]. In hypertonic assays it is lower than that obtained under isotonic conditions. The dependence of the activation energies with the method by which it is determined indicates that in conditions in which the bilayer conformation is preserved, the permeability coefficient would be a function of structural factors such as membrane density and hydration that can be altered by the osmotic shrinkage [3].

Since the mechanism of dehydration of glycerol is difficult to justify unless an interaction of the permeant with the hydrophilic regions of the membrane is taken place it is of interest to determine the changes promoted by glycerol on the surface of liposomes subjected to similar conditions to those employed in permeability assays.

The structural changes may be produced on the liposome surface by specific glycerol–phospholipid interactions, by shrinkage due to the osmotic contraction or by a combination of both factors. The combined effect of glycerol and shrinkage would be dependent on the phase state of the lipids in the bilayer.

For these reasons the interfacial properties of DPPC liposomes prepared in the different glycerol/glucose ratios in the absence and in the presence of osmotic gradients were determined using merocyanine 540 as an optical probe [12,13] below and above the phase transition temperature.

Materials and Methods

Dipalmitoylphosphatidylcholine was obtained from Avanti Polar Lipids Inc. A thin-layer chromatography gave a single spot when exposed to I_2 vapours. Therefore, lipids were used without further purification.

Liposomes were prepared using the method of Bangham [14]. A chloroformic solution of the lipids was evaporated under vacuum in a rotavapor. After a dry film was obtained in a round bottom flask, a buffer solution was added. Multilamellar liposomes were formed by gentle agitation above the phase transition temperature. In the experiments in which merocyanine 540 (MC540) was inside and outside, the buffer solution contained MC540 in a 10 μM concentration.

All the solutions were prepared in 10 mM Tris-HCl buffer (pH 7.4) and chemicals were of analytical grade. For the surface membrane studies merocyanine 540 was used as an optical probe [12,13]. After the liposomes were dispersed in the solutions of the desired tonicity, aliquots of a merocyanine stock solution in Tris buffer was added. After an incubation time of 30 min the spectra were run.

Merocyanine 540 is an optical probe showing one peak at 500 nm and other at 530 nm when dissolved in water and one peak at 570 nm when dissolved in a non-polar solvent [12]. In the presence of liposomes in the gel state in the aqueous suspension the merocyanine spectra are comparable to that corresponding to water. When liposomes are in the fluid state the spectra show a small shoulder at 530 nm and a defined peak at 570 nm. At the phase transition temperature the 570 nm peak increases without a complete disappearance of the peak at 500 nm.

The effect of glycerol concentrations and of the osmotic stress on the liposome surface properties was determined measuring the absorbance at 570 and 530 nm. The relative values of these peaks corresponds to the monomer-dimer equilibrium in the membrane [12].

The increase of the absorbance at 570 nm was taken with respect to a reference spectra. The reference spectra were obtained for each experiment applying the Gauss function to the peak at 530 nm. In these cases the absorbance increment at 570 nm was absent.

The osmotic gradients in experiments of Fig. 6 were established according to the relation

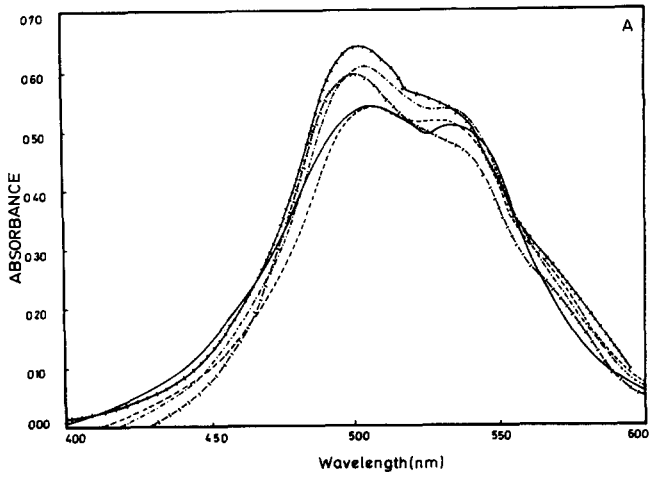
$$\Delta\Pi = RT(C_o\sigma_{glu} - C_i\sigma_{gly})$$

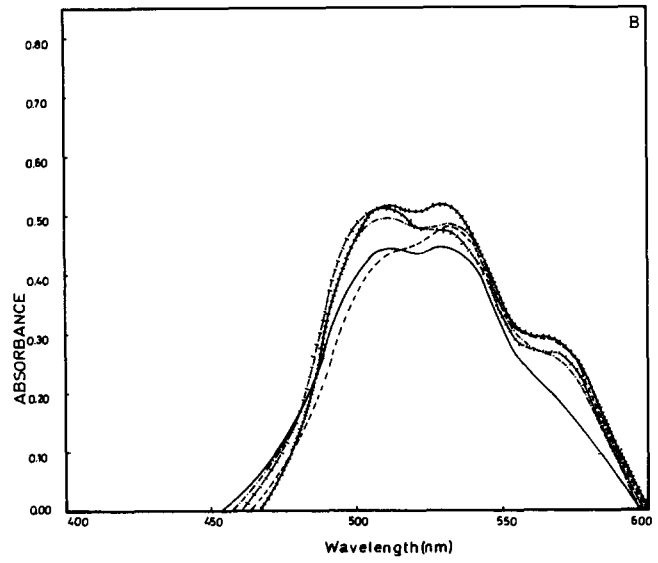
where C_o and C_i are the external and the internal concentrations of glucose or glycerol and σ_{glu} and σ_{gly} the reflexion coefficients for glucose (= 1) and glycerol (= 0.78), respectively. A possitive sign indicates a gradient promoting an outflux of water and hence volume contraction [11].

Results

The spectra of MC540 in the presence of liposomes in the gel state, which present a peak at 500 nm and another at 530 are changed when glycerol concentration is increased in the solution (Fig. 1). It is observed that at temperatures below the gel-liquid crystalline transition of DPPC (41° C) the absorbance at 570 nm increases with the glycerol/glucose ratio in which the liposomes were prepared.

The increase in the absorbance at 570 nm is paralleled by a decrease in that corresponding to 500 nm. This is more noticeable when temperature is increased from 25 to 35° C. This behavior is comparable to that obtained when the lipid bilayer is going through the phase transition. However, it must be noticed that the increase of 10 Cdeg in this range of temperature does not affect the form of the MC540 spectra when liposomes are prepared in buffer solutions containing solely glucose. This indicates that there is a specific effect of glycerol on the physicochemical properties of the liposomes which might be related to the affinity of the monomer dye with the lipid interfaces. Moreover, the changes in the spectra cannot be ascribed to a perturbation of the dye on the bilayer because if this would be





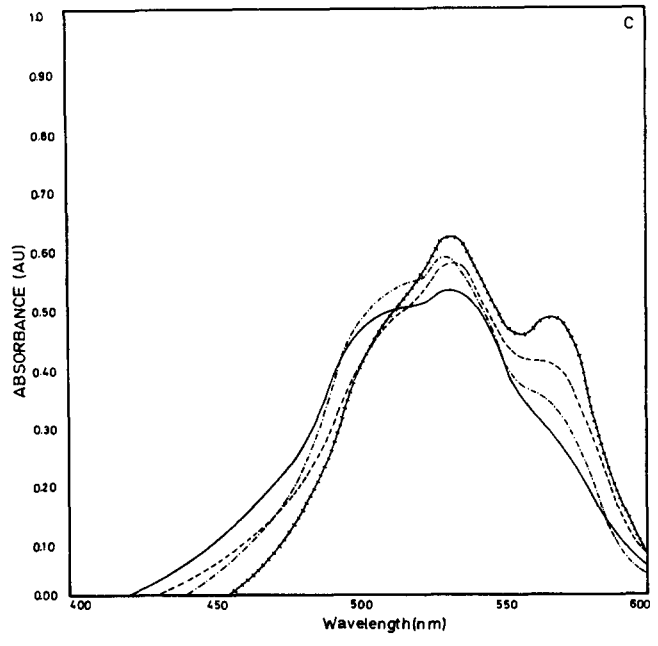


Fig. 1. Merocyanine spectra in the presence of DPPC liposomes prepared in glucose/glycerol solutions. (A) 25 °C, (B) 30 °C, (C) 35 °C. The different ratios of glucose/glycerol were : 1:0 (———); 0.75:0.25 (-·-·-); 0.5:0.5 (- | - | -); 0.25:0.75 (- - - -); 0:1 (++++). The total polyol concentration in the solution (glucose + glycerol) was in all cases 0.1 M.

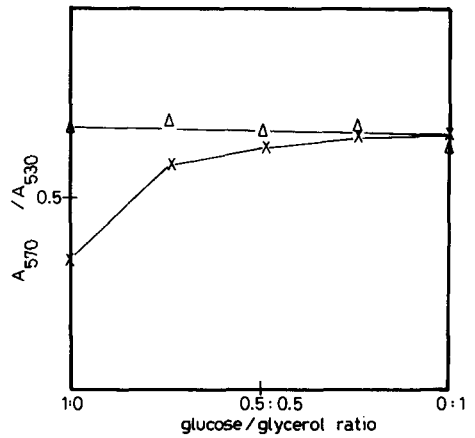


Fig. 2. Comparative effect on the A_{570}/A_{530} ratio of the glucose/glycerol ratio for liposomes with merocyanine in the outer solution (×) or in the outer and the inner solution (△). All measurements were done at 30 °C for a total polyol concentration of 0.1 M.

the case similar changes would have to be observed in buffer solutions with glucose.

The spectra of MC540 in Fig. 1 were obtained adding an aliquot of a stock solution to dispersions of liposomes prepared in the indicated glucose/glycerol ratios. Thus, it may be assumed that at that temperatures the 570 nm absorbance increases due to changes in the external surface properties of the different liposomes.

However, the simultaneous appearance of the peaks at 570 and 500 nm at 30 °C denotes that, at this temperatures, only a fraction of the lipids is in the fluid state as occurs in a bilayer at the phase transition temperature.

As it is well known, the permeability at the phase transition is several orders of magnitude higher than that corresponding to the gel or to the fluid state. Thus, the spectra at 30 °C in glycerol would denote a coexistance of gel and liquid crystalline phases affecting the permeability of the bilayer to MC. If this is the case, glycerol would enhance the penetration of the dye to the liposome interior.

The results of Fig. 2 and Table I indicate that the relative absorbances at 570 nm are dependant on the distribution of the dye between the inner and the outer compartments of the liposomes. Moreover, this distribution is influenced by the concentration of glycerol in contact with the bilayer.

In Table I, it can be observed that the A_{570}/A_{530} ratio is similar for liposomes prepared in glucose or

TABLE I

Absorbance ratio for DPPC liposomes with different distribution of glycerol, glucose and merocyanine between the inner and the outer media

All values were obtained at 30 °C.

Inner media	Outer media	A_{570}/A_{530}	
		merocyanine outside	merocyanine inside/outside
0.1 M glycerol	0.1 M glycerol	0.63 ± 0.05	0.67 ± 0.05
0.1 M glucose	0.1 M glucose	0.35 ± 0.05	0.68 ± 0.05
1 M glycerol	1 M glycerol	–	0.74 ± 0.05
1 M glucose	1 M glucose	–	0.74 ± 0.05

glycerol at different concentrations, provided that MC is inside and outside the liposomes.

In these cases, liposomes were prepared in buffer solutions of the polyols and the dye, which was not removed from the external media after the liposomes were formed.

However,a noticeable difference is observed when the dye is added to liposomes prepared in glycerol or glucose. In the first case, the absorbance ratio is comparable to that obtained with MC540 inside and outside. However, when liposomes are prepared in glucose the absorbance ratio is 40% less.

This is more clearly observed in Fig. 2. The greatest differences in the absorbance ratio induced by the glycerol/glucose levels are obtained when MC540 is added to the external solution of liposomes. When MC540 is present inside and outside no effect of the variations in the glycerol/glucose ratio is observed. Therefore, the data in Fig. 1 correspond to conditions in which MC540 is always outside.

The finding that increasing glycerol in liposomes with MC540 outside give similar ratios than that obtained with MC540 inside and outside, and that in the presence of glycerol it is indistinguishable to have

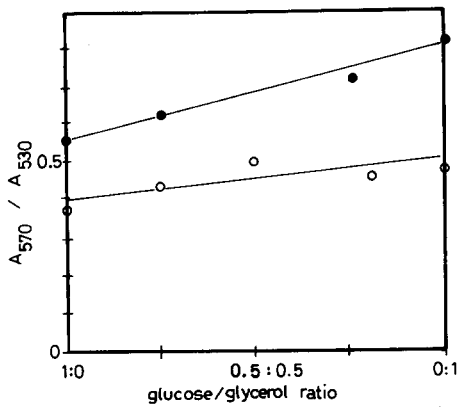


Fig. 3. Effect of glycerol on the A_{570}/A_{530} absorbance ratio. Liposomes were prepared in the glucose/glycerol ratio indicated in the abscissa for a total polyol concentration of 0.1 M and measured with merocyanine in the outer media at 25 °C (○) and 35 °C (●).

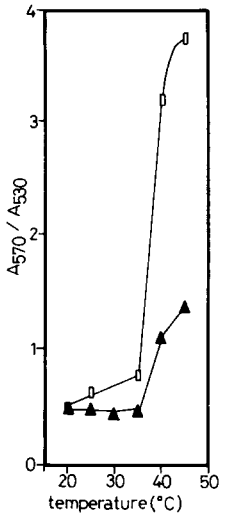


Fig. 4. Effect of glycerol on the onset of the gel-liquid crystalline transition temperature measured by the A_{570}/A_{530} ratio. Liposomes were prepared in 1 M glycerol (□) and 0.1 M glycerol (▲).

MC540 outside or at both sides would be an indication that glycerol changes the permeability of the bilayer allowing the distribution of the dye between the two media. This would be supported by the fact that glycerol induces a coexistence of gel and fluid regions.

The inspection of Fig. 1 in the light of the results of Fig. 2 and Table I indicates that at low temperatures and low glycerol concentrations MC540 is sensing the outer surface of the liposomes while the increase of such variables would give place to a redistribution of the dye.

In Fig. 3 it is observed that the increase promoted by glycerol is higher at 35 than at 25 °C. This would suggest that glycerol and temperature affects the bilayer properties in the same direction.

However, the presence of glycerol at concentrations of 1 M, is not able to displace the phase transition temperature that remains at 41 °C (Fig. 4).

This would be in agreement with the interpretation that glycerol is promoting fluid-like regions below the transition temperature and because of this the transition is broader in the presence of glycerol.

Considering that the greatest differences in the MC540 spectra are obtained when the dye is solely in the outer solution it is of interest to determine the changes in the spectra when the external surface properties of the liposomes are modified. This can be achieved by an asymmetric distribution of solutes such as glycerol or glucose.

In Fig. 5 the changes in the MC540 spectra obtained when the glucose or glycerol concentrations are higher outside than inside the liposomes are shown. Hence, they undergo an osmotic shrinkage. Under these conditions MC540 was added to the external media. In Fig. 6 it is shown the effect of the osmotic gradient on the absorbance at 570 nm obtained in the presence of liposomes in the gel and in the liquid-crystalline state.

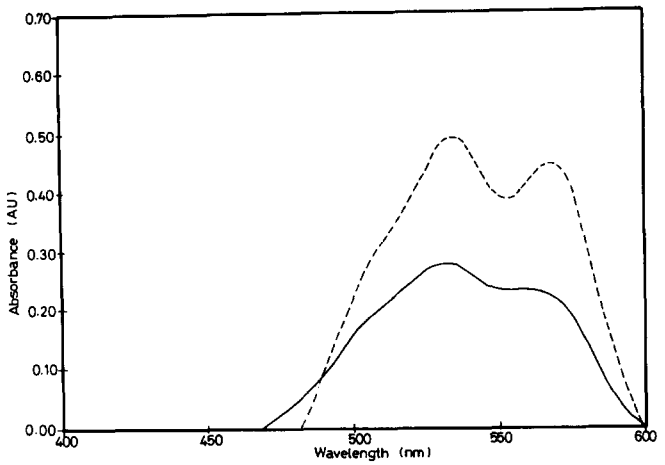


Fig. 5. Effect of shrinkage on the DPPC surface properties of liposomes in the gel state. Liposomes prepared in 0.1 M glycerol dispersed in 1.0 M glycerol (- - -). Liposomes prepared in 0.1 M glucose dispersed in 1.0 M glucose (———). Assays were performed at 25 °C.

It is observed that the absorbance at 570 nm decreases for liposomes in the liquid crystalline state when the osmolarity in the outer solution increases. It must be noticed that the osmotic response is greater when liposomes are filled with glycerol. At 25 °C, when the bilayer is in the gel state, the osmotic shrinkage produces an increase in the 570 nm peak.

These results indicate that the surface changes induced by the osmotic stress depend on the phase state of the lipids and on the type of solute with which the osmosis is created.

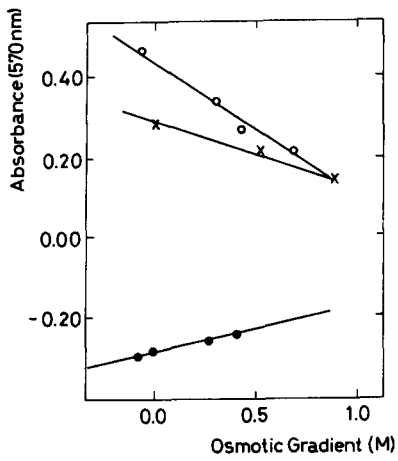


Fig. 6. Effect of the osmotic gradient on the surface properties of liposomes in the gel and in the liquid-crystalline state. Liposomes were prepared above the phase transition temperature in 0.1 M glycerol (○, ●) or in 0.1 M glucose (×) and dispersed in increasing concentrations of glucose or glycerol at 45 °C (upper curves) and at 25 °C (lower curve). MC540 was added to the external soliution after the osmotic equilibration of the liposomes. The absorbance at 570 nm was taken with respect to the peak at 530 nm. For details in the calculation of the osmotic gradient see Materials and Methods.

Discussion

The present results put into relevance that under isotonic and hypertonic conditions, noticeable changes on the bilayer surfaces are produced by glycerol. The properties of the lipid bilayer are not only altered by the osmotic stress to which it is subjected, but, in addition, it is affected by the concentration of permeant in the absence of gradients. The osmotic stress combines with the effect of glycerol giving opposite effects depending on the lipids are in the gel or in the liquid-crystalline state. In contrast, no effect are observed with isotonic solutions of glucose, a molecule for which lipid bilayers are known to be impermeable [11,14].

Permeability properties of lipid bilayers are determined by structural factors such as membrane density and hydration [3] which are a function of the phase state of the lipids. Thus, the changes observed with MC can be related to the variations of these properties. By means of different physicochemical variables such as temperature, osmotic stress or polar compounds, it is possible to induce the increase in the 570 nm absorbance.

At the phase transition, with high glycerol concentrations and when liposomes are shrunken, the increase in the absorbance at 570 nm takes place without a complete disappearance of the peak at 500 nm which corresponds to dimer in water solution.

Therefore, the changes observed in the MC540 spectra can be ascribed to modifications in the membrane structure induced by physical effects such as temperature and osmotic pressure or a chemical agent such as a polar permeant. These results indicate that different routes can be followed to obtain a coexistence of fluid and gel state in the lipid bilayer. At constant temperature, we can fluidize membranes in the gel state by provoking an osmotic collapse or increasing in isotonic conditions the permeant concentration.

For a given fluid/gel ratio, at constant osmotic gradient an increase in the fluid phase can be obtained by increasing the temperature. However, possibly to the increase in the restriction imposed by the volume contraction at this osmotic pressure, the fluid/gel ratio increases to a value much lower than that obtained in isotonic conditions. It is observed that at constant temperature, in the curve corresponding to 45 °C (Fig. 6) the decrease in the osmotic pressure increases the A_{570}/A_{530} ratio which is congruent with this interpretation.

At isotonic conditions, the decrease of temperature promotes the largest decrease in the A_{570}/A_{530} ratio. In contrast, under hypertonic conditions the decrease of temperature is not enough to condense all the bilayer surface to a gel state. This might be explained because defects which should disappear at the gel state cannot be annealed when the bilayer is in a compressed state.

Under these conditions, the permanence of defects would permit MC540 to enter the liposome interior. This point will be discussed below in the light of the results of Table I.

According to the relative magnitude of the peak at 570 nm and 530 nm it is clear that osmosis or glycerol do not induce a shift of the phase transition temperature (Fig. 4). However, the transition starts at lower temperatures indicating a broadening of the phase transition region in which gel to liquid domains can coexist. The coexistance of gel-fluid regions at temperatures below that of the phase transition temperature can give place to changes in the permeability of the bilayer.

In the absence of glycerol, MC540 would have access only to the outer interface, while at high glycerol concentrations MC540 can distribute between the inner and the outer media.

It is interesting to note that in all cases in which the 570 nm peak increases there is an increase in the bilayer permeability. That is, when the bilayer goes from the gel to the liquid crystalline state, and according to the results obtained with the dye inside and outside, when glycerol is added to a gel phase liposome. Moreover, taking into account that the osmotic shrinkage increases the 570 nm peak for liposomes in the gel state and that in the hypertonic method the activation energy is lower than in the isotonic one it can be said that this trend is also followed when the membrane is osmotically collapsed.

These results put into relevance that the experimental conditions, beside temperature, settled to determine permeability properties, that is, gradient of concentration and level of permeant concentration can affect membrane properties.

These results suggest that the kinetic barriers that a solute has to overcome to penetrate the bilayer can be modified by the action of the osmotic stress imposed by the permeant gradient to drive the flux and by perturbations of the permeant itself on the lipid interface. Hence, structural changes may be taking place in a comparable rate to that of permeation. These results are consistent with the visualization of the lipid membrane as a dynamic structure. For this, it is understood that the membrane phase can vary its properties by influence of the aqueous phase adjacent to it.

In connection with these results it can be mentioned that osmotic stress can induce topological changes in lipid bilayers affecting curvature and surface potential [14,15].

Therefore, permeability phenomena may involve changes in the surface free energy of the membrane which contributes to the penetration process.

Acknowledgements

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