

WATER IN BIOLOGICAL MEMBRANES AT INTERFACES: DOES IT PLAY A FUNCTIONAL ROLE?

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Abstract

The purpose of this review is to examine and discuss the ways in which water is organized at the interface of a biological membrane. The relevance of this structure to the surface properties and to the adsorption of proteins in membranes is also analyzed.

The approach is based on the idea that cell functions are confined to a restricted water media, the cell interior, in which the proximity of the membrane may be key to regulating the enzyme activity and the cell membrane permeability.

As the lipid bilayer is the structural base of cell membranes, the distribution of water in the surface sites of a phospholipid membrane is analyzed by means of Fourier Transform spectrometry. The polarization of water at the surface was looked into through the measure of surface potentials and the dynamics of the surface hydration by cyclic voltammetry. Modification of these properties by the replacement of water by polyol molecules such as trehalose and phloretin and by the insertion of aqueous soluble enzymes, has also been investigated.

Resumen

El propósito de este trabajo es analizar la organización del agua en la interfaz de una membrana biológica y su relevancia en las propiedades de superficie y en la adsorción de proteínas.

El enfoque consiste en considerar que la función celular está confinada a un medio restringido en agua, el interior celular; en el cual la proximidad de la membrana puede ser clave para regular la actividad enzimática y la permeabilidad.

Como la bicapa lipídica es la estructura básica de la membrana celular, los sitios de hidratación en la cabeza polar se analizan por medio de espectrometría infrarroja a transformada de Fourier. La contribución de la hidratación al potencial dipolar y la respuesta dinámica de monocapas de diferentes composiciones lipídicas se investigan mediante determinación de potenciales de superficie y voltametría cíclica.

La modificación de esas propiedades a causa del reemplazo de agua por polioles como trehalosa y floretina y por la inserción de proteínas acuosolubles, ha sido también investigada.

Abbreviations

PC: phosphatidylcholine
PE: phosphatidylethanolamine
PG: phosphatidylglycerol.
DMPC: dimiristoyl phosphatidylcholine
DPPC: dipalmitoyl phosphatidylcholine
DSPC: distearoyl phosphatidylcholine
DOPC: dioleoylphosphatidylcholine

Introduction

For many years, water has been for biologists as the canvas for painters. It is the support where the cell structures lie. While the aim has been to resolve the structure of membranes and proteins, the aqueous environment plays a passive, holder role.

However, numerous experimental results and theoretical considerations are driving the attention to a substantial role of water in the regulation of biological functions. For example, the drying of cells below a given level promotes irreversible changes and death. On the other hand, programmed cell death is concomitant with dehydration. That is why the question: "which of the water found in a biological system is engaged in the structural and functional properties?" becomes relevant.

The structural backbone of a biological membrane is the lipid bilayer, which consists of a variety of phospholipids: phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylglycerol (PG) and phosphatidylinositol (PI) among others. The bilayer is a 40 Å thick lamella including the region in which the phosphates and several groups esterified to it (choline, ethanolamine, glycerol, inositol), are exposed to the water media (Figure 1) [1,2].

Due to the complex composition found in cell membranes, the use of experimental model systems, of predetermined lipid composition, has proved to be a suitable way to explore the variety of physicochemical, electrical and mechanical properties of lipid membranes.

A short cut to these studies was the fact that lipids isolated from cells can stabilize, under certain conditions, in closed particles formed by lipid bilayers (multilamellar liposomes or unilamellar vesicles of different diameters). In turn, the amphipathic character of the lipids allowed the developing of a large number of studies in monolayers spread on the air-water or mercury-water interfaces. In the first case, the surface pressure/area isotherms provided information about the lateral compression and the thermodynamics of

the mixing of lipids in a bidimensional system [3-5]. Secondly, the application of a time-dependent potential across the lipid monolayer lead to information about the dynamic properties of the membrane components [6,7]. This review will deal with this methodology in section III.

Model systems have been extensively used to study phase properties, the compressibility, the permeability and the bilayer-no bilayer transition of lipid membranes [8-11]. The membrane properties at the phase transition have been ascribed to the coexistence of fluid and gel domains. The extension of these domains depends on the temperature and the lipid composition and has been studied by means of fluorescence and Brewster angle microscopies, both in the fluid and gel state [12-18].

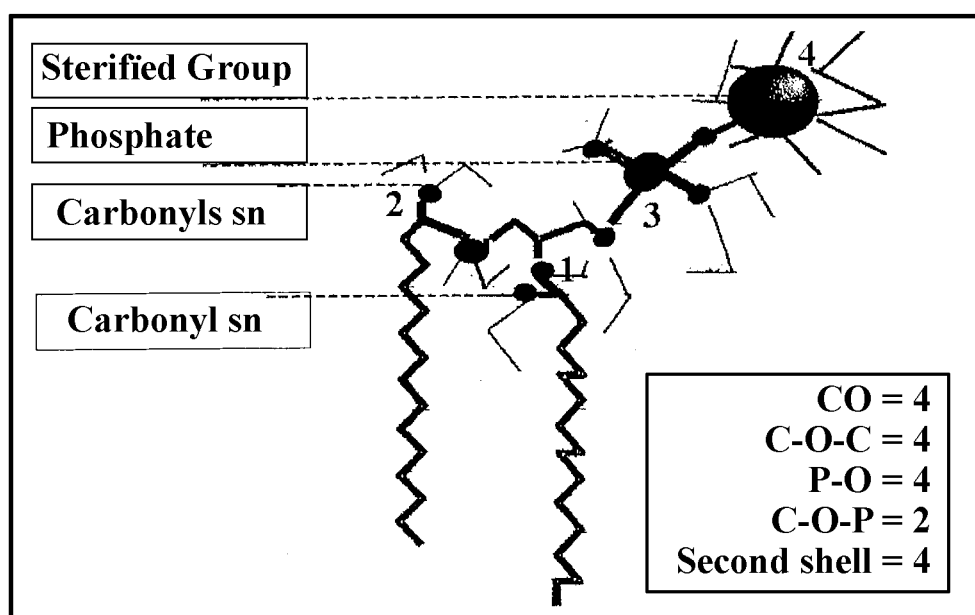


Fig.1. Organization of carbonyls, phosphates and esterified groups at the membrane surface with its hydration water molecules. 1. carbonyl network at the membrane plane, 2. carbonyls normal to the membrane plane, 3. phosphate ($P=O$) groups, 4. groups (choline, serine, inositol, glycerol) protruding into the water phase. The inset denotes the number of water molecules that may bind to different oxygen atoms.

Stress-strain transitions can be obtained in monolayers by changing the lateral surface pressure [19]. The lateral compression can force the lipid head groups to adopt different configurations, changing its orientation with respect to the plane of the membrane. In addition, a great deal of studies have been carried out in vesicles using well-characterized lipid compositions. The information obtained from investigation of these experimental systems indicates water is mainly localized around the polar head groups. A total amount of 18 to 20 water molecules per lipid has been found, depending on the lipid species, distributed in the carbonyl, C-O-C and phosphates bonds [20].

Part of this water is polarized and contributes to the surface potential, as will be

discussed in section II. In the bilayer conformation, the low dielectric permittivity of the hydrocarbon core ($\epsilon = 2$) makes the membrane an electrical insulator [21]. The orientation of water dipoles with respect to the plane of the membrane contributes to the dipole potential [22]. The combination of these two features gives rise to charge separation between the two sides of the bilayer, establishing a membrane potential.

The surface potential plays an essential role in the interaction of protein membranes in key biological phenomena such as membrane fusion, protein insertion and enzymatic activities. It is essentially a repulsive force, and its partial decrease may modulate the adhesion of cells, the adsorption of peptides and proteins to membranes and the penetration of amphiphilic molecules [23].

The presence of water in the membrane structure is crucial to maintaining its integrity and the permeability properties. Processes by which water is extracted from the membrane such as, dehydration, lyophilization or desiccation, affect the packing of the polar head groups and concomitantly the condensation of the acyl chains in the hydrocarbon core, leading to a solid crystalline state [24]. This state is rigid and fragile, with a low water permeability.

In addition, dehydration may produce a change in the relative areas of the polar groups with respect to the acyl chain region, affecting the topological conformation of the lipid arrangements. Thus, lipids that in normal conditions are stabilized in a bilayer may abandon this conformation to adopt a hexagonal phase, destroying the permeability barrier of the membrane and its ability to separate electrical charges [25].

With regard to the preservation of the bilayer structure and its properties in the absence of water, several polyhydroxylated compounds such as sugars and polyols have been found to buffer these changes. Among them trehalose, arbutine and phloretin are the most studied (Figure 2) [26-31]

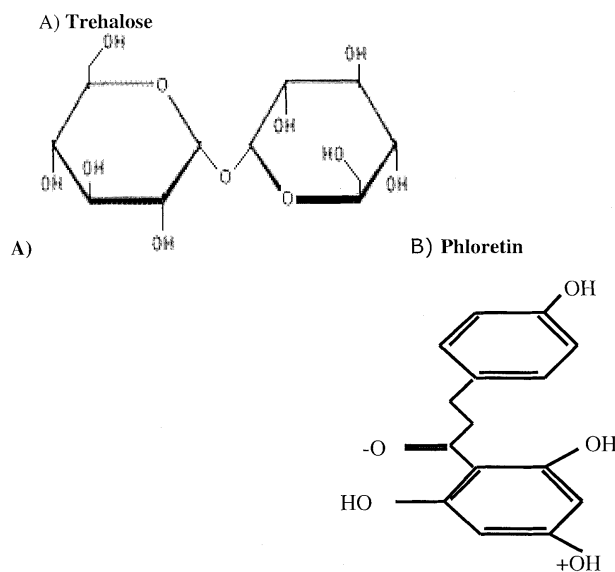


Fig.2. Molecular structure of trehalose (A) and Phloretin(B)

It must be emphasized that most of the studies about the lipid properties are centered in the phase behavior caused by temperature shifts. The phase transitions from gel to the liquid crystalline state and the influence of several factors such as additives in the aqueous media, cholesterol, protein insertion, etc., have been analyzed by several methods such as, differential scanning calorimetry, nuclear magnetic resonance, infrared spectroscopy, fluorescence and surface pressure/area isotherms [1,32-35].

Although it is known that water is located in the polar region, little is known about the organization and structure of water around the chemical groups of the polar heads, the distribution of the energy of interaction and the special organization of water dipoles around them, that may contribute to the dipole potential.

Thus, the modifications in the bilayer water interface by the presence of different types of phospholipids whose polar heads contain large dipoles, net charges and hydrogen bonding moieties, may be of significance for peptides and proteins to come into contact with the bilayer.

To get a better understanding of the distribution water at the interface of a lipid bilayer, it is of importance to know how water molecules are bound to the different polar moieties exposed to water at the bilayer interface. This distribution may affect the surface packing, the surface charge distribution and adhesion forces according to the type of phospholipids composing the membrane.

Specifically, the hydration of the phosphate groups and how it may be modulated by the presence of choline, glycerol or ethanolamine esterified to it, would be an indication of processes taking place in the water media adjacent to the membrane surface and its dependence on the lipid composition and surface properties.

To show this, we have correlated the state of hydration of different membranes with the adsorption and the activity of an aqueous soluble enzyme.

The properties of hydration of the lipid bilayer were measured by following the frequency of the vibration modes of the carbonyl and phosphate groups. The interfacial properties were measured in lipid monolayers on air-water or Hg-water interfaces determining the dipole potential, the surface lateral pressure and the response to a potential sweep.

I) SITES OF HYDRATION ON THE MEMBRANE SURFACE

Calorimetric measurements have shown that phosphatidylcholine hydrates with 5-7 water molecules in the gel state and 18-20 molecules in the liquid state [36].

According to Small Angle X ray Spectrometry (SAXS) of phosphatidylcholines, these water molecules are distributed in the region of the polar head where phosphates and cholines are located. In addition, a small penetration towards the carbonyl groups is also found [37]. In a first approach, we can distinguish three regions of hydration (see Figure 1)

- One is composed by two carbonyl populations in the sn_1 or sn_2 positions.
- The phosphate group..
- The group esterified to the phosphate (choline, ethanolamine and glycerol in PC, PE and PG, respectively).

The water level determines the stationary thickness and area per lipid of a lipid bilayer. The thickness of the water layer at each side of the membranes is around 10 Å thick [2]. Fourier Transform Infrared (FTIR) spectrometry is a powerful tool to explore the effect of hydration on the different polar groups of the lipid membrane. The effects of hydration and of compounds that may behave as substitutes for water on carbonyl and phosphate groups, will be analyzed.

Carbonyls

Carbonyl groups are present in the phospholipid molecules with two possible orientations: parallel and normal to the membrane plane (see Figure 1).

Hübner and Blume [34] showed that the populations of carbonyls with different levels of hydration are not congruent with the distribution of the sn_1 and sn_2 positions.

In fact, both, sn_1 and sn_2 carbonyls appear to have two different degrees of hydration.

This apparent incongruence suggests that carbonyls occupy alternatively two positions: one exposed to the water phase, likely the normal to the membrane plane and another hidden on the membrane plane. The trans bilayer distribution, in which a division between the interface and the hydrocarbon core is made, is in fact a time-averaged distribution. That is, it represents the probability of finding a particular structural group at a specific location in the bilayer or the number of groups per unit volume. The structural image is taken from diagrams of lipids with 5-6 water molecules per lipid [1]. However, the overall structure has only subtle changes with the increase in water content. Thus, fluctuations in water level may affect very slightly the overall thickness and the area per lipid of the membrane.

This dynamic picture of the hydration of the carbonyls is congruent with the fluctuations of hydration in the bilayer surface around a mean stationary value. In particular, it could be conceived that the fluctuations may be related to the water exchange between the membrane groups and bulk water and, hence, with the distribution in the energies of binding of the water molecule populations.

In this regard, fluctuations not affecting the average area and thickness could be those occurring in water molecules located in the second or third hydration layer, that is weakly bound to membrane groups.

The dynamic behavior of the lipid interface composed by PC's and PE's will be discussed again in section III.

Phosphates

Phosphate is a common chemical group in all the phospholipids composing natural membranes. It is linked to the acyl glycerol backbone and esterified to charged and uncharged chemical groups such as, choline, ethanolamines and glycerol.

Phosphate has been identified as the primary site of hydration of the phospholipids. The mobility of the phosphate increases with the hydration, reaching a constant value at around six water molecules per lipid. Thus, it is thought that the first six water molecules constitute the first hydration layer bound to the oxygen phosphates and ester bonds.

It has been shown that the asymmetric vibration of the PO_2 group is very sensitive to the hydration state of the bilayer. Thus, the hydration of the phosphate can be followed by the frequency shift of the $\text{P}=\text{O}$ asymmetrical mode. Exposure of lipids to dehydration by heat-drying and by osmotic stress promotes a shift of the $\text{P}=\text{O}$ frequency to higher values, denoting the displacement of water molecules (Table I).

Table I. Effect of osmosis and dehydration on the frequency values of the asymmetrical vibrational mode of PO_2 .

	PO_2 asym (cm^{-1})	Δ
DMPC dehydrated	1237	-26
DMPC in water	1211.14	
DMPC in PEG	1216	5.45

Δ represents the shift of the phosphate asymmetric mode when the lipids are dehydrated and when hydrated liposomes are subjected to an osmotic stress in polyethylene glycol 20% (PEG).

In the dehydrated state the frequency corresponding to the mode of vibration is 1237 cm^{-1} . Upon full hydration, (18-20 water molecules per lipid) the frequency decreases to 1211 due to the formation of hydrogen bonds with water molecules. When fully hydrated lipids are subjected to a hypertonic shock, such as by the addition of polyethylene glycol (PEG), the frequency increases nearly 6 cm^{-1} , indicating a small but significant, water displacement from the phosphate region.

In addition, hydrogen bonding compounds (Figure 2) may compete with water binding to carbonyls or to phosphate displacing the frequency to lower values. This indicates that the OH bridges of compounds such as trehalose or phloretin (polyphenol) are stronger than the H-bond of water with those groups. Trehalose, a dimer of glucose with the ability to form 10 hydrogen bonds, inserts in a lipid interface nearly normal to the bilayer plane [38].

This sugar binds to the carbonyl and to the phosphate groups, as derived from the frequency shifts in the FTIR spectra (Table II). Table III shows that phloretin only affects the asymmetric mode of vibration of the phosphate groups.

The shifts of the frequency mode of vibration for carbonyls and phosphates are correlated with the amount of water displaced by each of these groups from the lipid membrane (Tables II and III).

Lytic action and hydration sites

Mono acyl glycerophosphocholine (lyso PC), a conically shaped molecule, is able to destroy the bilayer conformation due to the difference of its molecular topology in comparison with that of phosphatidylcholine, of cylindrical symmetry. The ability of the

lyso PC's to disrupt lipid bilayer is affected by the type of sugar in contact with the interface. The insertion into the bilayer has been related to the presence of defects due to the mismatch between adjacent hydration shells, as it has been suggested to happen in the coexistence of gel and liquid crystalline phases [39].

FTIR data report that the addition of lyso PC at 18 °C to DMPC vesicles stabilized in water, decreases the frequency of the dehydrated population of the carbonyls without affecting the hydrated population [40]. Thus, the defects determining the lytic action can be related to the arrangement of the hydrogen bond network between the phospholipids. In consequence, the sites in which water binds to the phospholipids in a bilayer array, appears to be those where the lyso PC inserts.

Table II. Effect of trehalose on water activity and on the carbonyl and phosphate frequencies

Trehalose (mM)	Trehalose/lipid ratio	Water activity (%)	Carbonyl shift (cm ⁻¹)	Asymmetric Phosphate shift (cm ⁻¹)
0	0	100	0	0
0.05	2	50	-5	—
0.075	3	40	-5.4	-25
0.1	7	30	-7	-30

Table III. Effect of phloretin on the water activity and on the carbonyl and asymmetric phosphate vibration mode

Phloretin/lipid ratio	Water activity (%)	Carbonyl shift (cm ⁻¹)	Asymmetric Phosphate shift (cm ⁻¹)
0	100	0	0
0.25	92	4	-26
0.5	70	4	-30
1	64	4	-30

In the presence of sucrose, the action of the lytic molecules is enhanced, as shown by the lower lysoPC concentration needed to promote lysis with respect to that required in water. The critical concentration (c.l.c.) at which the bilayer is disrupted into micelles is 50 mM in water and decreases to 21 mM for the same lipid concentration in 0.1M sucrose. In contrast, the insertion of trehalose replacing water simultaneously at the carbonyls and the phosphates, does not cause severe changes in the lytic concentration with respect to hydrated lipids. In this case the critical lytic concentration is 41 mM for 0.1M trehalose. These results may be interpreted as a consequence of the extrusion of water molecules induced by sucrose, causing surface defects in which lysoPC can get inserted. In the case

of trehalose, water displacement is compensated with the insertion of sugar, thereby maintaining a similar surface structure. Defects are not formed and the action of the lyso PC is similar to that found in water.

In conclusion, water extrusion may cause defects in the arrangement of water layers, rendering the bilayer exposed to the penetration of amphiphilic compounds from the adjacent solution. Compounds that may substitute water can attenuate the formation of these defects. We have illustrated this point with lysoPC but the same reasoning holds for amphiphilic peptides or soluble proteins.

II) SURFACE (DIPOLE) POTENTIAL AND SURFACE TOPOGRAPHY

The insertion of lyso PC in PC bilayers and its disruption into micelles have been explained as a consequence of the presence of defects in the membrane due to the coexistence of gel and liquid crystalline states at the phase transition temperature or by the induction of defects by osmotic stress [41]. Details obtained from FTIR and from results of lytic action, suggest that defects may be a consequence of a redistribution of water among the chemical groups at the membrane surface.

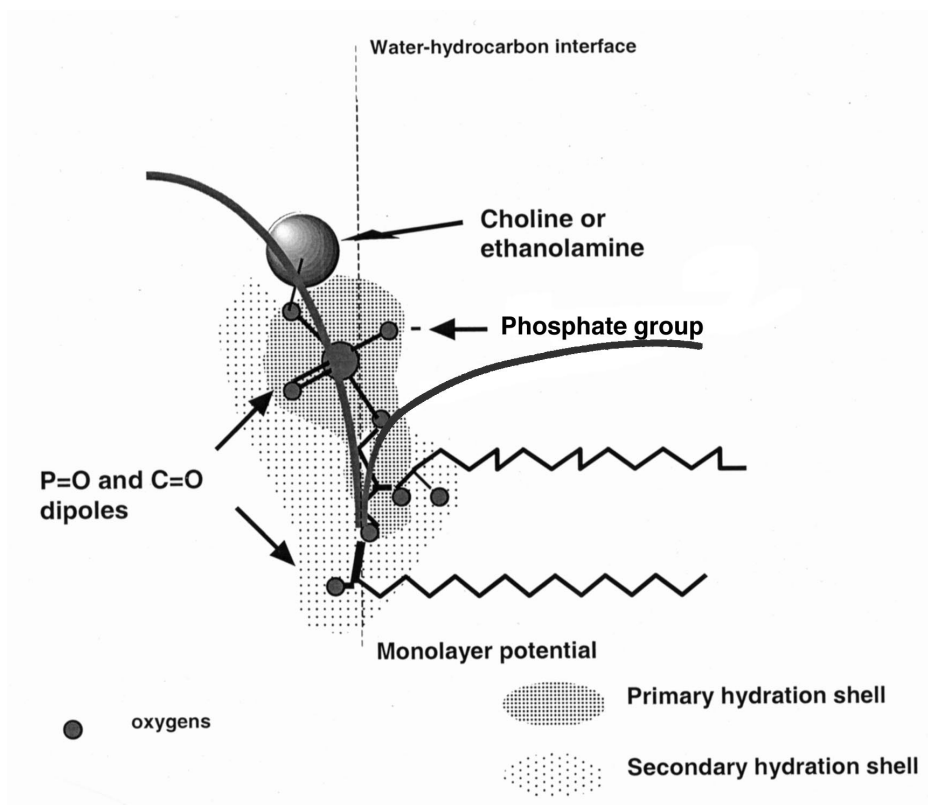


Fig. 3. Dipole potential in a lipid membrane interface. Water molecules, polarized by the C=O and P=O groups at the membrane interface, contribute to the dipole potential.

It has been shown that this water arrangement establishes a surface potential named the dipole potential (Figure 3). This potential modulates the penetration of peptides to the membrane phase, the permeability of hydrophobic anions, the permeation of water [42-44] and resists the membrane-membrane contact during adhesion processes. In this context, the potential provides a way to obtain a picture of the distribution of water at the lipid interface and its possible participation in the mechanism of relevant membrane phenomena, such as protein insertion.

A way to analyze different distributions of dipoles at the membrane interface is by studying the behavior of the surface potential as a function of the chain length, the presence of carbonyl groups and the influence of the group esterified to the phosphate.

The dipole potential of a lipid membrane is manifested between the hydrocarbon core of the membranes and the first few water molecules adjacent to the lipid head group (Figure 3). This potential is caused by the uniform orientation of the phospho-choline moiety, the carbonyl groups of the ester union and, to some extension, by the presence of polarizable groups in the membrane hydrocarbon phase [29, 45, 46].

Influence of chain length in lipid mixtures

The dipole potentials of monolayers of different DMPC/DPPC, DMPC/DSPC and DSPC/DOPC ratios show a non-regular pattern with the composition [47]. At the specific ratios of 0.33 and 0.66 of mixtures of lipids of different chain length in the gel state, the dipole potential is much higher than that predicted from mixtures of the pure components. The irregularities are attenuated when lipids of longer chain prevails in the mixture. The mixtures in which gel and fluid phases coexist or of lipids with similar chain length, show a continuous increase in the dipole potential when the component of the higher transition temperature prevails.

On the other hand, the electrokinetic potentials of vesicles show also singularities at similar compositions and conditions. These results suggest that when bilayers of short lipid chains are dopped with lipids of a longer chain (or viceversa) the topography of the surface promotes a defined water and ion distribution at the shear plane.

NMR experiments in pure lipids have shown that the head group is oriented parallel to the membrane plane [50]. However, in a mixture of lipids of different chain lengths, this regular array may change. Matching of chains of different lengths would result in phospholipid head groups located on different planes, allowing the head group to rotate around the C-O-P bond (see Figure 1). The positive end of the choline groups would be oriented towards the membrane phase thus allowing the adsorption of cations on the negative charge of exposed phosphates.

It has been shown previously that the mixture of DMPC and DPPC reaches +15 mV at a molar ratio of 0.33 with a minimum in dipole potential of 340 mV.

Taken together, dipole potential of mixed monolayers and electrokinetic mobilities of liposomes denote that lipid compositions show variations in the surface properties.

OH bonding compounds

The effect of the insertion of molecules, such as phloretin and trehalose, that interact with the moieties of the head group exposed to water, shown in the previous section, may decrease the dipole potential [31,48].

As shown in Table IV, a 20% phloretin decreases in 144 mV the dipole potential in phosphatidylcholine monolayers. This interaction is accompanied by the displacement of around six water molecules hydrating the phospholipids (Table III).

Different mechanisms have been described to explain the phloretin effect on the dipole potential. The primary effect of phloretin is thought to occur by the alignment of phloretin dipoles in opposite directions to those of the lipids [43]. It is also possible that phloretin may alter the structure of the interface changing the ordering of water or the orientation of the carbonyl esters. A decrease of the dipole potential of ether-phospholipids, i.e. those in which the fatty acid chains are bound to the glycerol by an ether union instead of an ester, has also been observed with phloretin [48]. This indicates that the effect of phloretin on the dipole potential is not related to carbonyl groups. In this regard, according to the FTIR measurements showed in Table III, there is no effect of phloretin on the ester carbonyl vibration modes. Thus, the decrease in dipole potential produced by phloretin on ester lipids is not due to H-bond formation or dehydration of the carbonyl dipoles.

Table IV. *Effect of phloretin and trehalose on the changes in dipole potential, water per lipid ratio, and shift of the carbonyl and phosphate vibrational frequencies.*

	Decrease in Dipole potential (mV)	Decrease in Water per lipid	Carbonyl mode Shift	Phosphate mode Shift
Phloretin (20%)	144	6	None	Decreases
Trehalose (0.1M)	50	11	Decreases	Decreases

Another explanation to the phloretin action also arises from FTIR measurements, showing that phloretin causes a pronounced downward shift of the frequency corresponding to the asymmetric vibration of the P=O groups of the phospholipids. This effect has been ascribed to the formation of hydrogen bonds between the OH groups of phloretin and the P=O. This interaction is concomitant with a decrease in the hydration of the lipids [31,48] from which it is inferred that the P=O–HO interaction is attended by water displacement. Thus, the action of phloretin on the dipole potential can be partly ascribed to the elimination of oriented water dipoles, hydrating the phosphate, from the lipid interface.

In the case of trehalose, the decrease in the dipole potential is lower in comparison to that produced by phloretin (Table IV). However, the data show that trehalose displaces more water from the lipid interface, both from carbonyls and phosphate.

Simulation studies have shown that trehalose may insert its own dipole into the bilayer interface, compensating the decrease produced by the displacement of water dipoles [38].

Ester phosphates, PE, PG and PC

As phloretin does not interact with carbonyls, the decrease of dipole potential is due to the elimination of polarized water from the P=O group.

It is of interest to correlate the phloretin action with the exposure of phosphate groups to the aqueous solution. In this regard, the head group arrangement of phospholipids, such as, phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol is distinct, due to the space requirement to orient the P-N dipoles. As a consequence, the hydration of the phosphate groups may change with the phase state of the lipids, the lateral surface pressure and the intermolecular interactions between adjacent molecules [34, 49, 50].

In PC's, the phosphate groups are linked to infinite zig-zag ribbons by water molecules of hydration. In this case, the choline end of the $-P-O-N^+(CH_3)_3$ group is laying toward the hydrocarbon phase, due to the hydrophobic character of the methylenes [50]. In contrast, the ammonium groups in PE link together with unesterified phosphate oxygen by very short bonds [36,51]. The ethanolamine groups is linked to adjacent phosphates producing a compact, rigid head group network at the bilayer surface. The $-N^+$ end of phosphatidylethanolamines (PE) seems to be oriented toward the water phase, forming hydrogen bonds between the phosphates and the amines of neighboring molecules, promoting a membrane packing. In phosphatidylglycerol (PG), the glycerol moiety appears to mimic the phosphate hydration [52].

With this basis, it is important to notice that the effect of phloretin on the dipole potential is modulated by the group esterified to the phosphoacyl glycerol.

The effectiveness of phloretin to decrease the dipole potential of monolayers in the fluid state is lessened by the moieties esterified to the phosphate group in the sequence choline > ethanolamine > glycerol. (Figure 4)

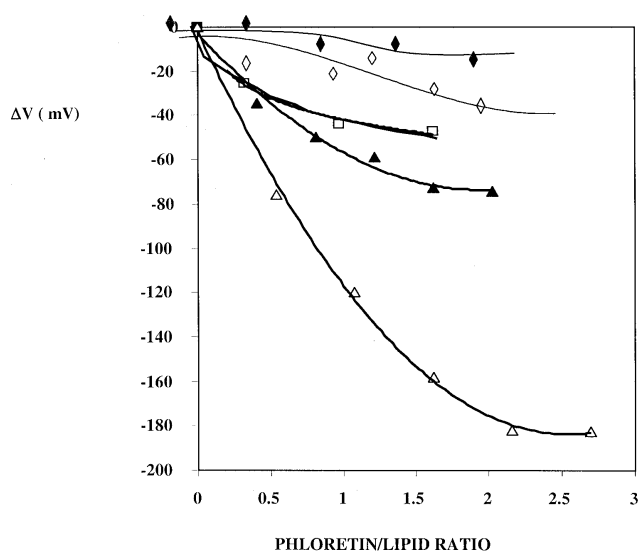


Fig. 4. Effect of Phloretin on the dipole potential of monolayers of different compositions in the presence of cholesterol. The relative changes in dipole potential (ΔV) are plotted as a function of the phloretin lipid /ratio for PC (Δ); PE (\blacktriangle); PG (\square), PE in the gel state without (\blacklozenge) and with cholesterol (\diamond).

Effect of cholesterol

In a similar way to the internal hydrogen bonds between different types of phospholipids, the intercalation of molecules acting as spacers (cholesterol, trehalose) could alter the access of phloretin to the phosphate groups and, in consequence, its effect on the dipole potential.

Changes of the dipole potential induced by phloretin were correlated with the packing of the lipids and with the formation of intermolecular hydrogen bonds. Phloretin does not affect the dipole potential in gel PE monolayers (Figure 4). However, phloretin decreases the dipole potential when cholesterol is included in PE membranes.

In conclusion, the changes induced on dipole potential by the interaction of OH groups are congruent with the displacement of water from the P=O groups and this is affected by the exposure of the group to the water phase.

III) DYNAMIC PROPERTIES OF MEMBRANE GROUPS WITH DIFFERENT DEGREES OF HYDRATION.

To further emphasize the dynamic character of the lipid interface in relation to fluctuations in hydration and group exposure, we will describe results obtained with bilayers subjected to different stresses. The osmotic stress imposed by the presence of macromolecules and the effect of an electrical potential across the lipid membrane will be analyzed.

Effect of the osmotic stress on the dynamic of surface groups

As shown in Table I, sucrose induces dehydration as derived from the increase in the frequency value of the asymmetric vibration mode of the PO₂ groups. This shift is paralleled to a displacement of four water molecules per lipid, which appears to be weakly bound water. Water displacement by the osmotic stress induced by sucrose would enhance the hydrophobic interaction of the choline moiety with the membrane phase, leading to a slight increase in the dipole potential of around 25 mV due to the exposure of the phosphate to the aqueous phase. This effect is concomitant to the formation of defects described above for the insertion of lyso PC.

The increase in the surface pressure due to a decrease in the water activity at the membrane interface, forces the dipole P-N to reorient with respect to the interface. That is, fluctuations in water activity may result in oscillations in the surface potential. In this case, water activity is the driving force coupled to the change in the electrical component of the membrane surface. Next section will discuss the effect of an electrical force on these surface groups.

PC and PE phase transitions in monolayers subjected to electric fields

As described in the introduction, a lipid membrane consists of a dielectric core, the hydrocarbon region, between two layers of polar head groups where water is polarized and ions are adsorbed.

Constitutive dipoles such as P=O and C=O groups along with polarized water, ac-

counts for the dipole surface potential. This potential can be modified by a lateral surface pressure [1, 4, 29, 44, 48, 53]. Thus, changes in the surface pressure may result in changes in the electrical surface properties.

On the other hand, when an electric field is applied across the membrane, charged groups and dipoles are expected to follow the potential change producing a structure reaccommodation and, in consequence, in the hydration state.

When the voltage applied corresponds to the energy required to reorient the groups, a change in capacitance or conductance is observed as a current peak. This procedure would give an insight into the dynamical response of the lipid interface organization.

To that end, phosphatidylcholine (PC) and phosphatidyletanolamine (PE) stabilized as monolayers on substrates such as Hg drops may be perturbed by a potential difference applied between the aqueous solution and the metal through the monolayer.

Adsorption of lipids on a mercury surface can be obtained by the passage of a mercury drop across a lipid monolayer formed at the air-solution interface. The metal surface a hanging drop mercury electrode (HDME) [6]. It is assembled on a vertical motion system, which allows the drop to run slowly through the solution surface inside the electrochemical cell. A micrometric screw adapted to the capillary allows drop size adjustment, and consequently, drop area regulation. A three-electrode cell was employed; the counter electrode was a Pt mesh of 1 cm² area, the reference electrode was a Ag|AgCl (3M) electrode. Lipid solutions were spread on the surface of buffer KH₂PO₄/K₂HPO₄ (Merck, PA). Solutions (pH 7.2) were de-aerated with nitrogen and kept under a constant flow of gas during measurements.

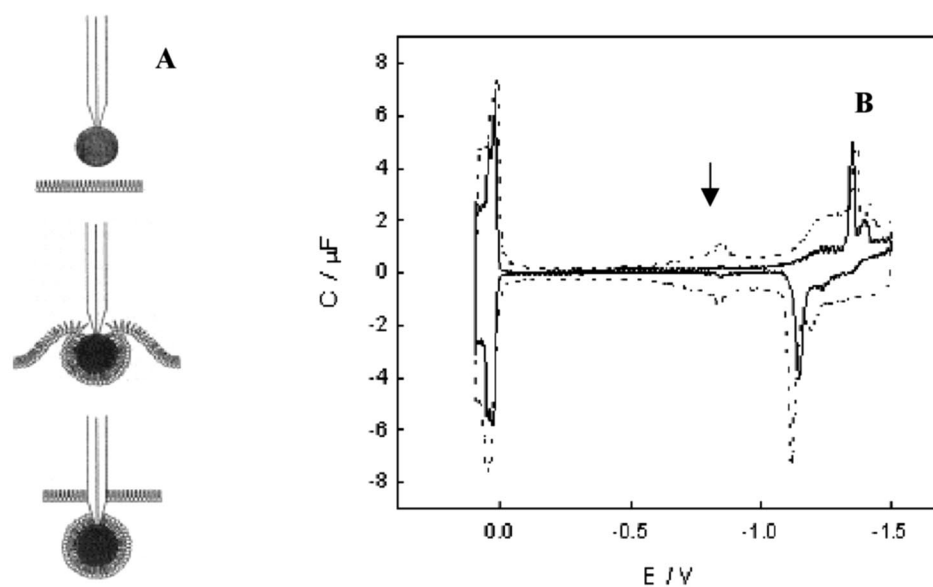


Fig. 5. Response of PC and PE monolayers to a linear potential sweep at a Hg/water interface. A) Formation of a monolayer on a Hg drop, B) Voltammogram of PC (full line) and PE (dotted line).

The arrow indicates the central peak at which a conformational change is taking place.

Figure 5 shows a scheme of the monolayer formed at the Hg-water interface and a typical voltammogram for PC. Different studies on PC monolayers on Hg drops, with and without cholesterol, have been reported [6, 7, 54].

Considerable differences among the peaks at the center of the voltammogram were found between PC and PE. This can be explained by the reorientation of the P-N moiety of the polar head groups in conditions in which the monolayer conformation is maintained. The bidimensional potential vs. pressure shows a maximum at -0.4V for 52 mN/m , close to that corresponding to DOPC in an air-water interface [54]. The pressure decreases gradually towards more negative potentials at which the transitions are observed. Thus, the appearance of the peak in the central region (at around -1.0 mV) is observed without abrupt changes in the surface pressure and is coincident with the appearance of punctual defects due to head group reorientations, without affecting the membrane cohesion.

The strong interaction of PE polar head groups, as a consequence of the hydrogen bonds between P⁻ and NH₃⁺ groups, seems to need more energy than the reorientation of the P-N⁺(CH₃)₃ groups, as suggested by the displacement of the potential of the central peaks to positive values.

The abrupt change in the potential of the central peaks at the transition temperature of DMPC (c.a. 24°C) can be related to a number of cooperative units that contribute to the transition (Figure 6)

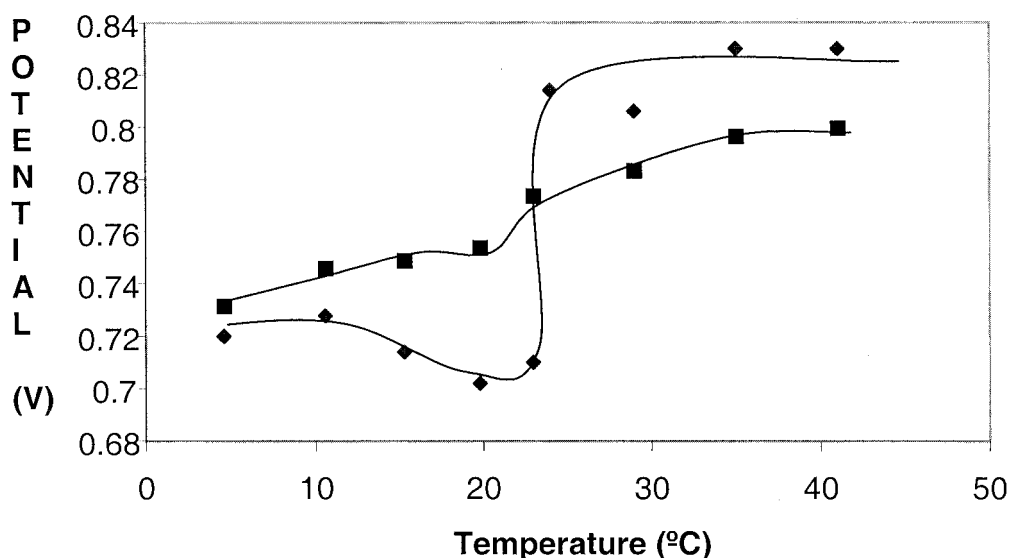


Fig. 6. Potential vs. temperature at the central and cathodic peaks. (◆) central peak (arrow in Figure 6B), (■) cathodic peak (at the right hand side of Figure 6B)

However, at more positive potentials the changes of the peak with temperature are much more attenuated. Thus, the growing of the defects may reach a critical point where they coalesce, forcing the lipids to abandon the monolayer structure. When that happened,

PE is favoured in comparison to PC as deduced from the lower potential values and may be explained by the well-known properties of PE to go into non-bilayer topological conformations.

A zero charge potential of +0.070V has been estimated for a DOPC monolayer adsorbed on Hg [54]. Thus, a more negative potential transition involves a higher energy. The cathodic peaks corresponding to DMPC appear in the region of -1.4V while those of DMPE are shown at -1.2V. Then, the formation of surface aggregates is a more energetic process for PC ending than for PE ending phospholipids. This last remark is derived from the fact that the cathodic region is related to the polar head groups.

The appearance of non-bilayer aggregates may be the reason why the change at the transition temperature is attenuated when the cathodic peak intensities are measured as a function of temperature. The cooperative units found at the central regions, i.e. when the monolayer is coherent, would be drastically reduced when the transition to non-bilayer aggregates appears at more negative potentials. Nevertheless, the transition is again centred at the value corresponding to the gel-fluid crystalline phase transition of DMPC, probably indicating that part of the lipids still remains as a monolayer.

The absence of carbonyls in ditetradecyl PC results in three cathodic peaks instead of one in DMPC. This may be interpreted to mean that the carbonyls in PC can interact with neighbour molecules by hydrogen bonds that add to those of the polar groups and hydrocarbon chains in the ether lipids, thus rendering the monolayer more stable. In contrast, ditetradecylPE and DMPE are practically identical. In this case, the contribution of the carbonyl group seems not to be important in PE's, probably due to the strong hydrogen bonding of the phosphates with the ethanolamines of adjacent molecules.

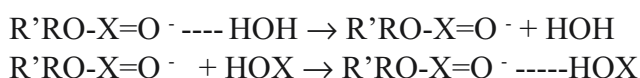
Summarizing, carbonyls are important for structural transitions in PC but not in PE.

In conclusion, at surface pressures where the monolayer is stable, the shift of the potential corresponding to the central peak seems to be due to a cooperative response of reorientation of the polar head groups as suggested by the sharp changes at the transition temperature.

It is interesting to point out that the information obtained from the voltammograms can be related to the reaccommodation of the polar moieties at the membrane interface. Thus, an energy profile might be necessary in order to determine the mean lifetime of the microstructure formed at the lipid interfaces.

IV) RELEVANCE OF MEMBRANE SURFACE PROPERTIES ON BIOLOGICAL FUNCTIONS.

Taking into account that the interaction of H bonding compounds from the aqueous solution with the P=O or C=O groups may follow a sequence such as:



where X stands either for C or P, it is reasonable to think that the step of dehydration may play a relevant role.

In the light of the information discussed in the previous sections, we may reconsider the model of the lipid interface. The lipid surface may be schematically described as a bidimensional layer in which lipids interact laterally along the carbonyls on the membrane plane. In this lattice, carbonyls normal to the membrane constitute the first layer of dipoles on which water is polarized. It should be taken into account that this lattice arrangement is an average conformation since, carbonyls fluctuate between the two positions with the associated water exchange (Figure 1).

Right above this layer, the phosphates are located, conforming the first layer of charges and a second layer of polarized water in the P=O groups.

The inclusion of the groups esterified to the phosphates results in a more complex and versatile structure.

Groups bonded to the phosphate are cholines, ethanolamines, glycerol, serine or inositol.

A picture of this surface observed from the water phase can offer several profiles, both in hydration and surface potential. In a region rich in PE, phosphate and amino groups would form a compact layer above the carbonyl, hindering the mobility of phosphates, its hydration and its interaction with OH of molecules such as phloretin. This layer is disrupted when cholesterol acts as a spacer as shown in Figure 5. The region is hydrated with less than 4 water molecules.

In PC rich regions, the hydrophobicity of the choline group will pull it towards the membrane phase. This orientation would make the phosphate group, and hence its charge, to be exposed to the water phase. This region has 18-20 water molecules per lipid, and OH groups from solutes such as phloretin or trehalose have access both to phosphates and carbonyls.

The exposure of the phosphate to water may be blocked by the glycerol moiety. In this case, the OH groups of the glycerol would mimic the water of hydration, thus decreasing the access of phloretin to the phosphate.

Groups, such as serine and inositol, that are strongly hydrophilic, would protrude from the surface of the membrane into the water phase.

According to this picture it is immediate to realize that the adsorption and insertion of a molecule from the water phase need to overcome several energetic barriers of different nature depending on membrane composition.

Effect of the membrane surface on protein interactions

Proteases show considerable changes in its enzymatic activity when trapped in reverse micelles [55]. These results indicate that the environment affects the catalytic activity of water - soluble enzymes. This finding has considerable importance on the understanding of the regulation of enzymes, since protease activity could be modulated by the interaction with lipid surfaces of different properties. For instance, in the intracellular activity of protease of the HIV it could be of importance to know if the accessibility to the membrane surface could be a factor affecting activity and / or inhibition [56]. If this were the case, regulation of this interaction could be a way to reduce or increase the proteolysis. As derived from the analysis in the previous sections, water restricted media are directly

related to the hydration state of the membrane structure since phospholipids stabilize in bilayers by hydrating their polar head groups [57]. In addition, steric hindrance, electrostatic forces and hydration of the phospholipids play significant roles in protein-membrane interactions [58].

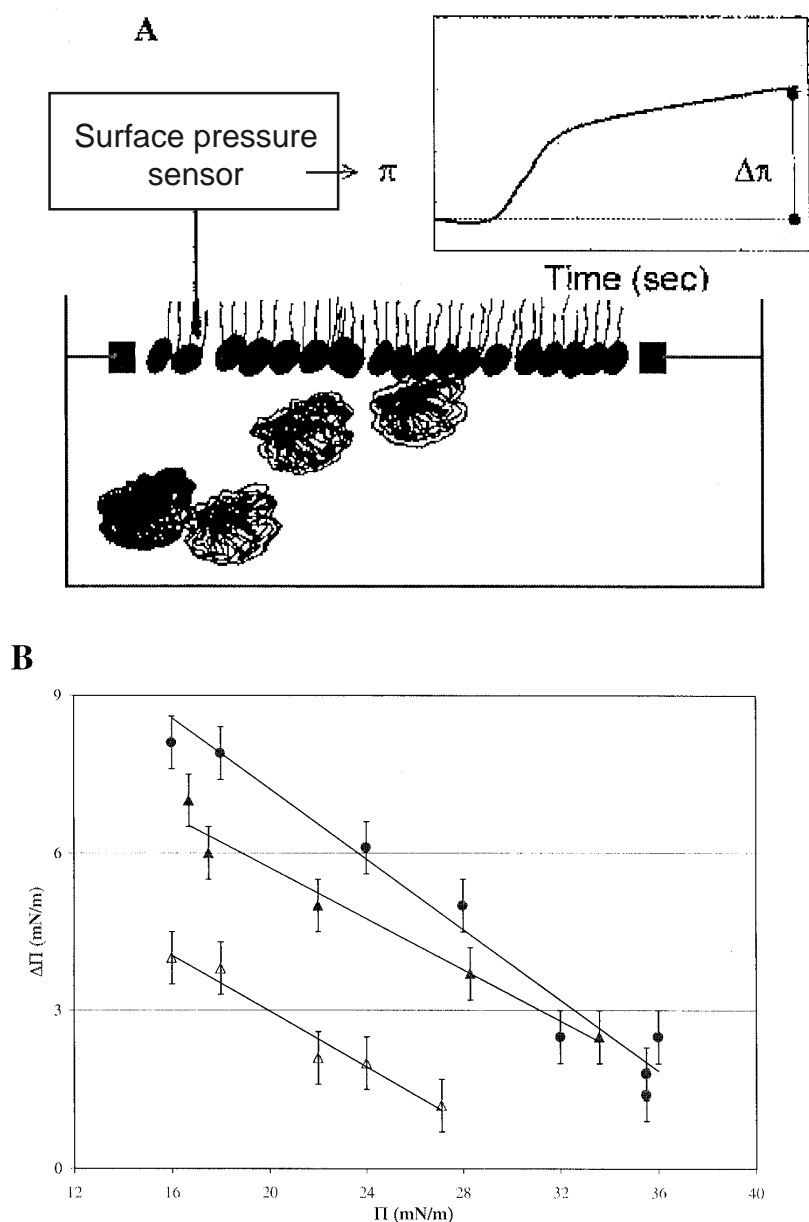


Fig. 7. Interaction of a protease with lipid monolayers.

A) Measurement of surface pressure: schematic representation.

B) Increase of surface pressure vs. initial surface pressure on monolayers of DMPC (◆), DMPE (Δ) and DMPC (\blacktriangle).

Different polar head groups and hydration levels determine different values of the surface and dipole potentials [48]. Hence, changes in the lipid species may affect protein-membrane interactions by a combination of electrostatic and non electrostatic forces.

It has been shown that an aspartyl protease of *Mucor miehei* strongly interacts with lipid membranes containing positively charged groups [59]. However, at high ionic strength, the interaction was found to be dependent on different lengths and saturation of the fatty acid chains of the phosphatidylcholines, denoting the participation of non-electrostatic forces as well.

The interaction of an aqueous soluble protease (from *Mucor miehei*) with monolayers composed by dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidyl ethanolamine (DMPE), and dioleoylphosphatidyl choline plus phloretin (DOPC:Phlo) (1:1) can be followed by the changes in the surface pressure measured at constant temperature as a function of the initial lateral pressure of the monolayers (see inset to Figure 7A).

It is observed that at low lateral pressures the major changes were induced by protease on monolayers in the fluid state. However, for the same lateral pressures, the effects were much lower when the monolayer was composed by DMPE or by DOPC:Phlo (Figure 7B).

As already mentioned, phloretin affects the hydration of the phosphate groups decreasing the dipole potential [48]. This effect is not observed in DMPE whose phosphate group is hydrogen bonded to the amine group [60]. Moreover, the interaction of the protein with PC monolayers is notably reduced in the presence of phloretin. Thus, it may be postulated that the phosphate groups should be exposed in order to facilitate the protein adsorption.

In agreement with the adsorption pattern and the penetration behaviour on PC's, the proteolytic activity remains unchanged. However, in PE or DOPC/phloretin membranes, the activity is enhanced. The activity changes may be related to the intensity with which the protease interacts with the different lipids. Hence, the interaction decreases when the phosphate group is not freely exposed as in PC/phloretin and DMPE bilayers.

That is, interfaces with low water activity increase the enzyme activity, in coincidence with results reported for reversed micelles.

Conclusions

Two main sites of hydration can be identified by FTIR: phosphate and carbonyls. The phosphate group is a sensor of hydration in lipid bilayers. The frequency of the asymmetric vibration of phosphate is displaced to lower values by osmotic stress induced by PEG or sucrose.

Carbonyl hydration seems to be perturbed by osmosis, causing defects in the membrane packing were amphiphilic compounds can get inserted.

OH forming compounds bind to phosphate or to phosphate and carbonyls. The presence of choline, ethanolamine or glycerol in the molecule affects the binding to the phosphate as shown with phloretin.

A profile of water distribution can be done on the membrane plane according to the

lipid species with different hydration. PC's appear to be the region rich in water, and PE's the less hydrated. This hydration affects water replacement by OH compounds, the dynamics of the ethanolamine and choline groups and the insertion of aqueous proteins.

The present results suggest the possibility that the complex lipid composition of lipid membranes may have an expression in terms of water organization at the membrane surface. This organization would give rise to domains in water dipole arrangements, fluctuating around equilibrium positions. Further studies should give information about the mean life time of such domains, its extension and participation in biological phenomena.

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