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Dynamics of Proton Diffusion within the Hydration Layer of Phospholipid Membrane†

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ABSTRACT: The diffusion of protons at the immediate vicinity of (less than 10 Å from) a phospholipid membrane is studied by the application of the laser-induced proton pulse. A light-sensitive proton emitter (8-hydroxypyrene-1,3,6-trisulfonate) was trapped exclusively in the hydration layers of multilamellar vesicles made of egg phosphatidylcholine, and the protons were dissociated by a synchronizing laser pulse. The recombination of the proton with pyranin anion was monitored by time-resolved spectroscopy and analyzed by a diffusion-controlled formalism. The measured diffusion coefficient is only slightly smaller than the diffusion coefficient of proton in bulk water. Modulating the width of the hydration layer by external pressure had a direct effect on the diffusibility of the proton: the narrower the hydration layer, the slower is the diffusion of protons.

A precise understanding of proton diffusion on the membrane–water interface is central to experimental observations regarding chemiosmotic proton flux (Chiang & Dilly, 1987). The diffusion of protons between source and sink, located on a membrane, is a sum of two pathways: the lateral proton diffusion through the hydration layer and the bulk diffusion (Nagle & Dilly, 1986). The fast equilibration of protons between surface and bulk (Nachliel & Gutman, 1984) implies that even if the observation time is limited to a few microseconds after protons were released (from a surface source), their reaction (with a surface sink) will be dominated by diffusion through the bulk phase.

In recent years Prats et al. [(1987a,b) and references cited therein] studied the diffusion of protons at a water–monolayer interface. According to their model the proton diffusion at

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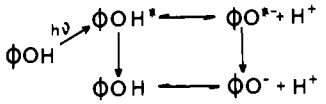
Scheme I



an interface is regulated by three processes: (1) The protons have to penetrate into the hydration layer which is separated from the bulk by an energy barrier. The energy for this step is supplied by the mechanical stirring of the solution (Prats et al., 1985). (2) Within the hydration layer the protons diffuse much faster than in the bulk. The diffusion coefficient, due to the ordering of the water, is ~20 times larger than in bulk phase. (3) The protons are withdrawn from the hydration layer into the bulk at a rate that is accelerated by addition of buffer (Prats et al., 1987a,b).

The first assumption is implausible. In a condensed medium, like water, molecules exchange energy by collision at a rate of 10^{15} s^{-1}. In such systems the energy distribution is Boltzmannian, and stirring cannot provide a molecule with extra energy to pass a potential barrier. The latter assumption is an underestimation of the high velocity of bulk surface proton exchange. Gutman and Nachliel (1985) demonstrated that acid–base equilibrium between bulk and surface is reestablished within 1 μs after a perturbation. Thus the claim of Prats and his colleagues that protons can remain in the hydration layer for many minutes seems unlikely (Kasianowicz et al., 1987a,b). Our reservations about the conclusions of Prats and his colleagues reemphasize the necessity to obtain an accurate, reliable value of proton diffusion within the hydration layer of lipid membranes.

The hydration layer is an inhomogeneous matrix. Water molecules that are in contact with the phospholipid head groups are stabilized (with respect to bulk water) by ~1 kcal/mol, but the interaction energy decays exponentially with distance from the membrane with a length constant comparable to the dimension of a water molecule. At a distance of ~10 Å, the interaction energy is already well below kT [Lis et al., 1982a,b; Parsegian et al., 1979; Parsegian et al. (1986) and references cited therein].

To observe the diffusion of a proton, exclusively in the hydration layer, we must have a system where this phase will be well insulated from bulk water. A useful model system consists of multilamellar vesicles where the lipid layers are separated by ultrathin (~25-Å) water layers. Furthermore, the width of these water layers is controllable by external forces. Under applied osmotic pressure water molecules with lower potential are removed, and the remaining layer will be narrower and more ordered.

The diffusion of protons within hydration layers was measured by trapping 8-hydroxypyrene-1,3,6-trisulfonate (pyranin), in MLV under conditions where the inner water body [with a diameter of ~500 Å (Lichtenberg & Markello, 1984)] is free of the dye. The proton dissociation from pyranin is driven by a short laser pulse (see Scheme I). The excited dye (ϕOH^*)[1] dissociates within 100 ps, and the excited anion (ϕO^{*-}) decays within a few nanoseconds to the ground state (ϕO^-) characterized by a strong absorption band. The time-resolved measurement of the absorbance of ϕO^- monitors its diffusion-controlled reprotonation.

[1] Abbreviations: PC, phosphatidylcholine; ϕOH and ϕO^-, acidic and alkaline forms of the proton emitter; ϕOH^* and ϕO^{*-}, first electronically excited states of the respective forms of proton emitter; $D_{(2)}$ and $D_{(3)}$, two- and three-dimensional diffusion coefficients; $[\phi O^-_{(2)}]$, two-dimensional concentration of ϕO^- in units of mol/cm².

Table I: Pyranin Content of Phosphatidylcholine Multilamellar Vesicles Swelled in the Presence of Pyranin

	pyranin/lipid (mol/mol)	
	coevaporated PC–pyranin mixture swelled in water	PC swelled in aqueous pyranin
MLV	0.075	0.016
freeze–thawed MLV[a]	0.043	0.0135

[a] MLV were diluted 1:100 in 10 mM Hepes, pH 5.7, subjected to eight cycles of rapid freezing and thawing, and collected by centrifugation.

The diffusion coefficient of protons was calculated from these dynamics by a quantitative analysis based on equations for diffusion-controlled reactions in either a two-dimensional (Hardt, 1979) or a three-dimensional space (Smulchowski, 1916; Gutman, 1984, 1986). We find that proton diffusion within the hydration layer is modulated by the intensity of the hydration forces. In unstressed multilamellar vesicles, where the equilibrium width of the hydration layer is 25 Å, the diffusion coefficient is essentially that measured in bulk water. Under osmotic pressure which compresses the membranes to ~10 Å apart, the diffusion coefficient is smaller.

Our observation is in accord with the fact that ordering of water molecules slows the diffusion of protons [Huppert and Pines (1985) and references cited therein]. The measured decrement of D^H in the hydration layer is rather small, indicating that the ordering effect of hydration forces does not immobilize the water for a period much longer than that needed for proton exchange between neighboring molecules.

Materials and Methods

Preparation of MLV. Egg yolk phosphatidylcholine (Sigma grade VII-E) was dried to a thin film in a flask and swelled (12 h, 37 °C) in the presence of 2 mM pyranin, pH = 5.7 (250 mg of H_2O/mg of PC). The suspension was washed by centrifugation with 10 mM MES buffer (pH = 5.7) until the supernatant was free of pyranin.

Kinetic Measurements. MLV containing pyranin were suspended in 10 mM Mes, pH 5.7, to a nominal dye concentration of ~30 μM. The dilute suspension was placed in a four-face quartz cuvette (4 × 10 mm) at the crossing of two laser beams: an excitation beam (337 nm, 0.5 MW/cm² pulsing at a frequency of 10 Hz (10 ns full width of pulse at half of maximal intensity), and a monitoring beam (457 nm of an argon ion laser). Modulation of the monitoring beam intensity, due to transient formation of the pyranin anion, was measured with 10-ns resolution as described elsewhere (Gutman, 1986; Nachliel & Gutman, 1988).

Results

Distribution of Pyranin in Multilamellar Vesicles. Two procedures were used for trapping pyranin in multilamellar vesicles. In the first one, the dye and lipid were dissolved in methanol, the solvent was removed in a rotary evaporator, and the film was swelled in water. Vesicles made by this method contained a large quantity of pyranin, but ~45% of it was removed after a few cycles of freeze–thawing (see Table I). This fraction of dye is attributed to pyranin trapped in the central water core of the multilamellar structure (Schwartz & McConnell, 1978).

The second method we used was to dry the lipid in the absence of dye and then swell it in an aqueous solution of pyranin. The trapping efficiency of this procedure is much smaller, yet practically no dye is released by freeze–thaw

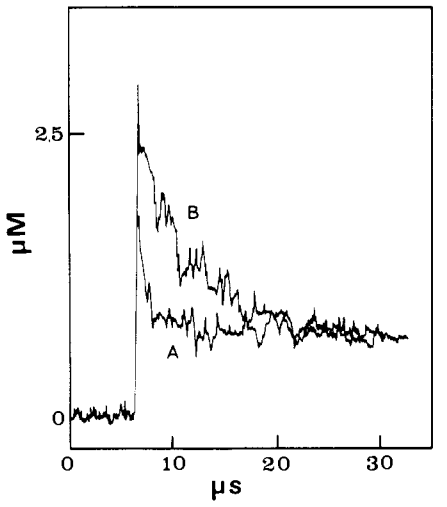


FIGURE 1: Transient absorption measurement of reprotonation of pyranin anion trapped within the hydration layers of phosphatidylcholine multilamellar vesicles. (A) Vesicles suspended in 10 mM Mes buffer, pH 5.7. (B) Vesicles under osmotic pressure applied by 1.6 *m* sucrose (4.7×10^7 dyn/cm^2) in 10 mM Mes, pH 5.7.

cycles. Formation of multilamellar vesicles by swelling of dry film is progressive. The outer layers of the vesicles are formed first, and inner ones follow in time as water percolates inside. During this process the hydrophilic dye cannot cross the many lipid layers. Its entrapment within the water layer is through transient breaking of membrane continuity as the vesicles swell in size. For this reason swelling in aqueous pyranin solution produces multilamellar liposomes where the dye is present mostly in the outer water layers and hardly any is present in the inner core.

Comparison of the dye/lipid ratio of the two freeze–thawed preparations implies that when added in the swelling medium, not all hydration layers are loaded with the dye (~30%) [see also Gruner et al. (1985)].

As we are interested in preparation where the dye is located exclusively in the hydration layer, throughout this study we trapped the dye by adding it to the swelling medium.

Time-Resolved Measurements of Proton Diffusion in the Hydration Layer. Photoexcitation of pyranin by a laser pulse initiates the reactions described in Scheme I. The incremental dissociation is monitored at 457 nm, where ϕO^- has a strong absorbance (E_{457} = 24 000 M^{-1} cm^{-1}). The time-resolved absorbance measurement (see Figure 1) corresponds with the reprotonation of the dye. Line A was measured in MLV suspended in 10 mM Mes, pH 5.7. This buffer concentration is sufficiently high (Gutman et al., 1985) to suppress proton transfer by pyranin adsorbed on the external surface of the vesicles (Clement & Gould, 1981). Whatever we measure in its presence is a reversible proton dissociation of dye exclusively trapped in the hydration layers. Line B was measured with vesicles suspended in 1.6 *m* solution of sucrose. Under this osmotic pressure (4.6×10^7 dyn/cm^2) the water is removed from the hydration layer and its width decreases from 25 to ~14 Å [see data of Parsegian et al. (1979)].

The dependence of the recombination rate on the width of the hydration layer is shown in Figure 2. As the width decreases from 25 to ~10 Å, the rate of reaction is slowed by 20-fold.

On the basis of these results, we conclude that water molecules that are immobilized by strong interaction with the membrane, i.e., within the first hydration layer, are a poorer matrix for proton diffusion than more remote layers. Furthermore, due to the symmetry of the hydration layer between

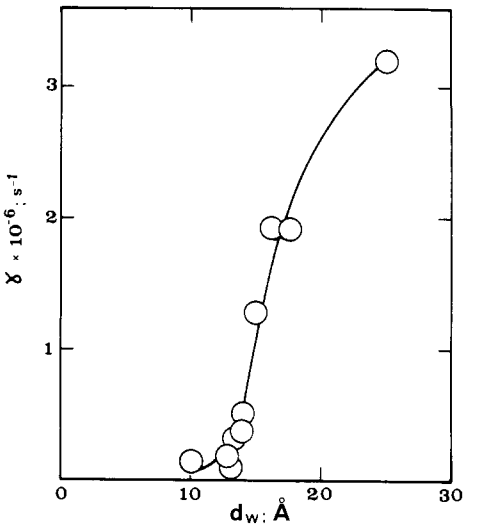


FIGURE 2: Dependence of the observed rate of reprotonation (γ) on the width of the hydration layer. The experiment, as in Figure 1, was carried out with the same vesicle preparation under varying osmotic pressures. The width of the hydration layer d_w was calculated from the data of Parsegian et al. (1979).

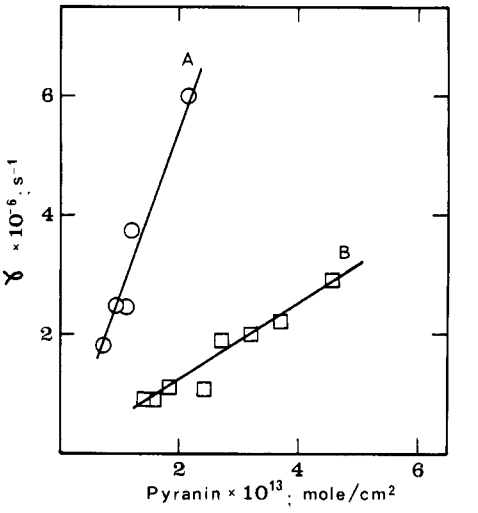


FIGURE 3: Dependence of the observed rate of reprotonation on the two-dimensional concentration of pyranin anion in the hydration layer. The rate constant (γ) was calculated from the experiments carried out as in Figure 1. The concentration of the anion was calculated from the amplitude of the transient and the dye/lipid ratio of the preparation. The magnitude of the amplitude was varied by modulation of the excitation pulse energy by glass filters. (A) Measurements were carried out in the absence of sucrose. (B) Measurements were carried out in the presence of 0.57 *m* sucrose (1.5×10^7 dyn/cm^2).

two membranes, the central layer of water molecules will have the highest proton conductivity.

Calculation of the Two-Dimensional Diffusion Coefficient of the Proton. For quantitative calculation of the proton's diffusion coefficient we assumed that proton diffusion is limited to the central water layer. Thus the geometry of the system is of a two-dimensional space. For such a model there is an analytic expression (Hardt, 1979) that relates the measured rate constant (γ) with the two-dimensional diffusion coefficient [$D^H_{(2)}$ given in cm^2/s].

$$D^H_{(2)} = \frac{\gamma}{2\pi N[\phi O^-_{(2)}]} \ln \frac{1}{r_{AB}\sqrt{\pi N[\phi O^-_{(2)}]}} \quad (1)$$

According to this equation the relaxation rate will vary, nearly linearly, with the two-dimensional concentration of the pyranin

Scheme II

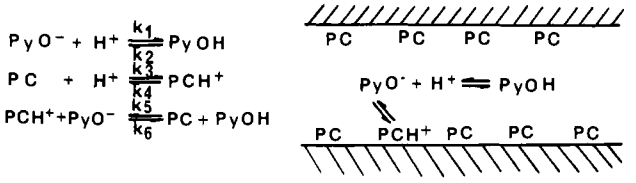


Table II: Kinetic Constants of Proton-Transfer Reactions and Physical Properties Measured in Hydration Layers in Phosphatidylcholine Multilamellar Vesicles

	sucrose (m)	
	0	0.57
osmotic pressure (dyn/cm^2)	5×10^5 [a]	1.5×10^7
width of hydration layer[b] (Å)	25	15
rate constant of reprotonation (M^{-1} s^{-1})	$(5 \pm 1) \times 10^{10}$	$(2 \pm 1) \times 10^{10}$
diffusion coefficient of proton (cm^2/s)	10×10^{-5} [c] 2×10^{-5} [d]	4.4×10^{-5} [c] 1×10^{-5} [d]
rate constant for ϕO^- reaction with PC–H (M^{-1} s^{-1})	$(1 \pm 0.5) \times 10^9$	$(4 \pm 0.5) \times 10^8$
diffusion coefficient of ϕO^- (cm^2/s)	2.2×10^{-6} [c] 0.5×10^{-6} [d]	0.9×10^{-6} [c] 0.2×10^{-6} [d]
viscosity of water in hydration layer (cP)	1.6–7	3–12

[a] Contribution of 0.02 osM of buffer. [b] Estimated from the data of Parsegian et al. (1979). [c] Calculated from the rate constant by assuming total electrostatic screening by ions within the hydration layer and r_{AB} = 6 Å. [d] Calculated from the rate constant by assuming no electrostatic screening of the interacting ions and r_{AB} = 6 Å.

anion [$\phi O^-_{(2)}$] (given in units of mol/cm^2).

As the initial conditions of our experiment maintain very low ϕO^- concentration, we replace [$\phi O^-_{(2)}$] by [$\Delta\phi O^-_{(2)}$] (the incremental dissociation of ϕOH by the laser pulse). In this form eq 1 predicts a nearly linear dependence of γ on $\Delta\phi O^-$.

Figure 3 depicts the result of a set of measurements where $\Delta\phi O^-$ was controlled by the intensity of the excitation pulse. Line A was measured in unpressed MLV, while line B was measured under a pressure of 1.5×10^7 dyn/cm^2. From the slopes of the lines we calculated the two-dimensional diffusion coefficients to be $D^H_{(2)} = (2.5 \pm 0.5) \times 10^{-5}$ cm^2/s for membranes spaced at ~25 Å and $(0.65 \pm 0.1) \times 10^{-5}$ cm^2/s at a separation distance of 15 Å (lines A and B, respectively).

Determination of the Three-Dimensional Diffusion Coefficients and the Local Viscosity. The density of the lipid head groups in the the interlamellar aqueous phase is very high. The average space per phospholipid head group (in uncompressed MLV) is ~950 Å^3, which is ~1.8 times smaller than the space available for a molecule in a 1 M solution. The pK of phosphatidylcholine is low (pK = 2.25) (Nachliel & Gutman, 1988), yet at such high density repetitive trapping of protons by the phospholipid head groups will delay the recombination of protons with ϕO^-.

The contribution of this mechanism to the measured event can be subjected to quantitative evaluation, using a numerical solution of coupled, nonlinear, parametric differential equations which represent the reactions of pyranin anion, phosphatidylcholine, and protons as defined in Scheme II.

The solution of the parametric differential equations is a unique set of rate constants that reconstruct the experimental curves [for a detailed explanation of the parameter search and listing of differential equations see Yam et al. (1988)].

Figure 4 depicts the reconstructed dynamics of proton recombination as measured in unstressed multilamellar vesicles (frame A) or under 1.5×10^7 dyn/cm^2 (frame B). The rate constants and the corresponding physical parameters derived from them are listed in Table II.

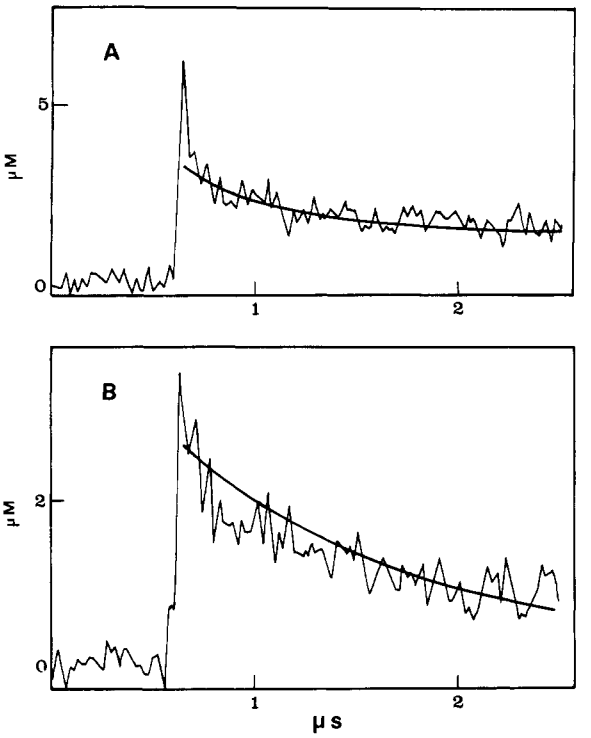


FIGURE 4: Numerical simulation of the dynamics of reprotonation by solution of differential equations. The smooth, continuous curve is the dynamics predicted by the rate constant listed in Table II and defined in Scheme II. (A) Experiment carried out in the absence of sucrose. (B) Experiment measured in the presence of 0.57 m sucrose (1.5×10^7 dyn/cm^2).

The magnitudes of the second-order, diffusion-controlled rate constants of the reactions were analyzed by the Debye–Smoluchowski equation, which relates the rate constant with the dimension of encounter complex, electrostatic interaction, and diffusion coefficients of the reactants.

$$k_{AB} = \frac{4\pi N r_{AB} \sum D}{1000} \frac{\delta}{e^\delta - 1} e^{\delta[r_{AB}\kappa/(1+r_{AB}\kappa)]} \qquad (2)$$

The first term, the rate of encounter (k_{en}), relates the rate constant with the sum of the diffusion coefficients ($\sum D$) and the sum of the radii (r_{AB}).

The second term modulates the rate of encounter by the electrostatic force where

$$\delta = Z_1 Z_2 e_0^2 / \epsilon k T r_{AB}$$

For pyranin anion in aqueous solution δ = −4.7.

The last term in eq 2 is the electrostatic screening by the ionic atmosphere. When the screening is minimal, we can approximate $k_{AB} = k_{en}\,[\delta/(e^\delta - 1)]$. If the screening is intensive, then $k_{AB} \simeq k_{en}$. As we have no accurate estimation for the effect of ionic screening within the hydration layer, we estimated the diffusion coefficient between these two margins.

Discussion

The basis of this study is the accurate measurement of the rate constant of a diffusion-controlled reaction of proton with pyranin anion. This reaction has been extensively measured (Forster & Volkers, 1975; Gutman, 1984; Pines & Huppert, 1985), and the diffusion coefficient of protons, derived from the rate constant, is essentially identical with that measured by classical conductivity measurements.

The physical system we used for our measurements, multilamellar vesicles, is also well characterized. The width of hydration layer and the potential of water in the midplane of

the hydration layer have been measured and corroborated by more than one method (Lis et al., 1982a,b; Parsegian et al., 1979; Israelachvilli & Mara, 1986).

The equation of Hardt (1979) was used to determine the two-dimensional diffusion coefficient in a set of experiments where one of the initial conditions parameters (size of perturbation) was a controllable term. The resulting value of $D^{H}_{(2)}$ is in sharp contrast to the estimation of Prats et al. (1987a,b). The diffusion in the interface is definitely slower, not faster than in the bulk phase. When the vesicles were partially dehydrated by osmotic pressure, $D^{H}_{(2)}$ became smaller. At a pressure of 1.5×10^7 dyn/cm^2, the width of the hydration layer of egg phosphatidylcholine is $\sim$15 Å, i.e., six water molecule layers between the two lipid membranes. The diffusion coefficient so close to a surface (6×10^{-6} cm^2/s) is about 5% of the diffusion coefficient in bulk water.

The analysis of the results according to the Hardt equation is subject to certain criticism. The scenario does not consider the capacity of the phosphocholine head groups to trap temporarily the protons, delaying the reprotonation of ϕO^-.

To account for this mechanism, we applied a different analytic approach. The reactants were assumed to be randomly dispersed (in a three-dimensional space) where both H^+ and ϕO^- diffuse with their own diffusion coefficients [$D^{H}_{(3)}$ and $D_{(3)}{}^{\phi O^-}$], while phosphatidylcholine is immobile. The concentration of the reactants (in molar units) was that corresponding with the average space available for the species.

The reactions defined by Scheme II were combined into the coupled, nonlinear differential equations given in the Appendix of Gutman (1984). A numerical solution of the equation (depicted as the reconstructed dynamics in Figure 4) yielded the rate constants and physical parameters listed in Table II. The outcome of these calculations is not in contrast with the value estimated by Hardt equation. The value of $D^{H}_{(3)}$, in uncompressed vesicles, is actually the value measured in bulk water with the uncertainty margin due to difficulties in correction for the ionic screening. The lower value 2×10^{-5} cm^2/s is obtained if we assume full electrostatic attraction between ϕO^- and H^+. The higher value 10×10^{-5} corresponds with the assumption that the ionic atmosphere completely screens the charges of the reacting species. The actual value of $D^{H}_{(3)}$ falls somewhere within this narrow range. This diffusion coefficient measured in the unpressed hydration layer is not surprising, considering that in these vesicles the potential of water in the midplane is that of the bulk. The viscosity of the water as measured for the same system is also close to that of bulk water. This measurement indicates that the viscosity of the water is practically constant up to the plane of shear which, for water, is located $\sim$1 Å from the interface surface (Israelachvili, 1985).

Under osmotic pressure the diffusion of proton is slowed by 50%, which corroborates the tendency derived from the Hardt equation. Thus we conclude that the proton conductivity of the hydration layer decreases as the potential of the residual water molecule increases.

In past years the water at the membrane or protein surface was described by figurative terms like "soft ice" or "flickering iceberg" structures. Our measurements are not in accord with this suggestive nomenclature. It is more likely that water at the interface maintains its liquid property practically all the way to the surface.

Registry No. H^+, 12408-02-5; water, 7732-18-5.

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